ISSN 0001-6772

cta hysiologica candinavica

blished monthly for the
andinavian Physiological Society

ol 107 No 1 September 1979

APSCAX 107 (1) 1–96 (1979)

Editorial office

Acta Physiologica Scandinavica
Karolinska Institutet
S-104 01 Stockholm

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erformance of the hypertrophied left ventricle in pontaneously hypertensive rat. Effects of changes in reload and afterload

EDDY NORESSON SVEN-ERIK RICKSTEN MARGARETA HALLBÄCK NORDLANDER and PETER THORÉN

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NORESSON E., RICKSTEN S. E., HALLBÄCK-NORDLANDER, M & THORÉN P Performance of the hypertrophied left ventricle in spontaneously hypertensive rat. Effects of changes in preload and afterload. Acta Physiol Scand 1979 107 1–8. Received 13 June 1978. ISSN 0001-6772. Department of Physiology University of Göteborg, Sweden.

Isolated hearts from adult spontaneously hypertensive rats (SHR, Okamoto 1969) with established hypertension were investigated in an antegrade perfusion apparatus where preload and afterload could be varied independently Frank Starling curves were constructed at constant afterloads ranging from 50 mmHg to 150 mmHg. As earlier reported, the SHR hearts exhibited a rightward shift of their Frank Starling relationships compared to those from the normotensive control hearts, though visible only at afterloads up to about 100 mmHg At higher afterloads the SHR hearts performed significantly better then the NCR ones as their maximal stroke volume was significantly greater compared to that of controls. Thus left ventricular hypertrophy obviously increases the work capacity of the heart, though at the cost of an altered Frank-Starling relation dependent on the reduced diastolic compliance. For such reasons the myocardial hypertrophy in established SHR hypertension must be considered physiologic adaptation and not degenerative phenomenon, though naturally degenerative processes may later become superimposed

Key ords Primary hypertension, spontaneously hypertensive rat, left ventricle Frank Starling relation, rightward shift, preload, afterload, maximal cardiac performance

Left ventricular hypertrophy is a general finding in established hypertension The increased tension development per myocardial fibre when the left ventricle contracts against an enhanced aortic pressure is considered to be the key stimulus for this hypertrophy When ventricular hypertrophy has balanced off the elevation of arterial pressure, i.e afterload' the increased muscle mass contributes to the increased total tension development so that tension per transverse section area of contractile elements is again normalized (cf Meerson 1969). In the spontaneously hypertensive rat (SHR, Okamoto 1963) being one of the best animal analogues for essential hypertension in man, the first trace of an increase of left ventricular mass appears as early as 3 days after birth and may here contain an element of hyperplasia as well as hypertrophy (Weiss & Lundgren 1978). Later in life left ventricular weight in per cent of body weight increases in due proportion to the increased arterial pressure (Farmer et al 1974) A similar structural adaptation to increased pressure load takes place also within the systemic precapillary resistance vessels where it appears as an increased wall thickness in relation to the internal radius in SHR and in human essential hypertension (Folkow et al. 1973)

Left ventricular hypertrophy as seen in hypertension, has been considered as a principally physiologic adaptation (Hallbäck, Isaksson & Noresson 1975) but it has also been regarded as a more or less degenerative phenomenon (Averill et al 1975) It has by the former group been shown that an increased filling pressure is required to produce equal stroke volumes in isolated perfused hypertrophied hearts from SHR compared with hearts from normotensive controls (NCR) Thus the Frank Starling relationship of hypertrophied hearts was at lower filling pressures displaced to the right of controls in proportion to the degree of left ventricular hypertrophy However at higher filling

Table 1 *Mean arterial pressure heart rate body weight and left ventricular weight in per cent of body weight in adult SHR and matched NCR*

Values presented are mean ± S E Levels of significance are given

	Blood pressure (mmHg)	Heart rate (beats/min)	Body weight (g)	Left ventricular weight in *per cent of body weight*
SHR ($n = 17$)	148±1	374±?	323±1	0.304±0.005
NCR ($n = 17$)	*100±1*	356±2	376±2	0.226±0.001
P	0 001	n.s	0 001	0 001
SHR/NCR	1 48	1 05	0.86	1.35

pressures these differences were no longer at hand This suggests that the rightward shift of the Frank Starling relationship of the hypertrophied hearts is not a consequence of degenerative phenomena since the differences then would be further accentuated at high filling pressures It is instead *suggested to be due to an altered relationship be*tween enddiastolic pressure and enddiastolic tension in the more thickwalled SHR ventricles (cf Hallbäck 1975)

The present study was undertaken to further investigate these matters and to study how the work performance of hypertrophied hearts differs from that of normotensive control hearts when the cardiac work is altered over a wide range by changing either filling pressures (preload) or peak aortic pressures (afterload) For this purpose isolated beating hearts from adult SHR and from normotensive control rats (NCR) were perfused antegradely in a system where preload and afterload could be varied independently In this way Frank Starling curves could be constructed for different levels of constant afterloads

METHODS

A imals 17 4–5 months old male SHR (weight around 325 g) and 17 NCR of the same age and sex but slightly heavier (weight around 375 g *Table 1) were included in the study* They were fed a standard pellet diet and had water ad libitum

Pr parations Mean arterial pressure (MAP) measurements were performed one week prior to the actual experiments During light ether anesthesia a PE 50 catheter was inserted into the tail artery The rats were allowed to wake up and MAP was then measured during steady state conditions. At the day of experiment the rats were heparinized (50 IU/100 g b w) and 15 min later anesthetized with ether *After a midline incision the thymus* and the pericardium were removed the large vessels cut and the heart was immediately placed in icecold saline which within 5 stopped its activity The aortic transection was made at least 4 mm above the coronary entrances so that no interferance with coronary flow would occur when the heart was mounted in the perfusion apparatus The aortic cannula which had an outer diameter of 3 mm, was inserted and tightly ligated Within 40 s after the excision the heart was perfused via the aorta and the coronaries with a non-recirculating Krebs-Henseleit bicarbonate *buffer* Contractions started within seconds after the beginning of the initial washout period The preperfusion lasted for 6–8 min. Meanwhile a major pul*monary vein was cannulated with the angeled cannula* shown in Fig 1 while all other pulmonary veins were ligated

From now on the heart was *instead perfused* via left atrium in an antegrad fashion with a recirculating perfusion fluid (Fig. 1) Krebs-Henseleit bicarbonate buffer gassed with 95% O_2 and 5% CO_2 was used as perfusate (Krebs & Henseleit 1932) The volume of the recirculating perfusate was held constant (120 ml) by a reservoir (Fig 1) The perfusate contained (mM) NaCl 118 KCl 4 7 $CaCl_2$ 2.5 $MgSO_4$ 1.2 KH_2PO_4 1.2 $NaHCO_3$ 25 NaEDTA 0 5 and glucose 14

Perfusion apparatus A modification of the antegrade perfusion apparatus originally described by Morgan and Neely was used (Morgan et al 1965 Neely et al 1967 cf Isaksson 1972) as shown in Fig 1 The apparatus consisted of a 130 cm long oxygenating chamber with a micropore filter at the bottom (cf Isaksson 1972) A peristaltic pump with an output of 450 ml/min was collecting the perfusate leaving the oxygenating chamber and *pumped it to the top of the chamber and to the atrial* bubble trap The preload could be varied by changing the height of atrial bubble trap over the heart level. The connecting tube (Tygon tube *inner diameter 6 mm)* between the heart and the atrial bubble trap contained another small bubble trap because small bubbles could occasionally slip through the first trap The preload i e left atrial pressure was recorded via side tube connected to a Statham 23 DC transducer which was placed at the level of the heart and writing on a Grass polygraph Compensations were made for the pressure fall in the tube between the transducer and the heart

The heart was *pumping against a modified Starling re*sistor (Fig) By adjusting the air pressure in the balloon the aortic pressure could be maintained constant at any desired level *The aortic pressure was recorded on a* Grass polygraph via a Statham 23 DC transducer (Fig 1)

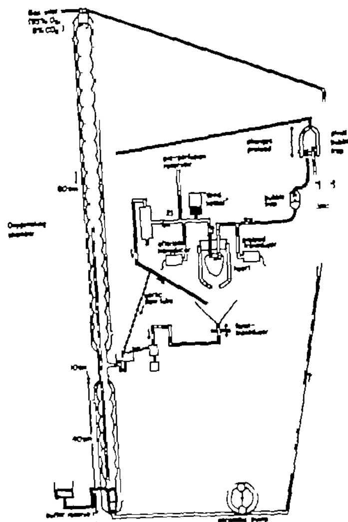


Fig 1 Schematic illustration of the antegrade perfusion system where preload and afterload can be varied independently

The aortic pulse pressure could be varied by changing the amount of air in the 'Windkessel' (normally 2 ml) and was held between 10–15 mmHg. This implies that the mean pressure levels were 5–8 mmHg lower than the peak values. Changes in peak aortic pressure were taken as expressions of changes in left ventricular afterload. The coronary flow dripping from the heart and the aortic flow were diverted into funnel placed on top of Grass FT10 force transducer. An electronic valve was placed on the outlet from the funnel, closing it off during 6 s, every 10th s, so that an intermittent and exact flow registration was obtained by means of the periodic weight increases then recorded by the force transducer. In this way total cardiac output (CO) was registered but when the aortic output went directly to the oxygenator (Fig. 1 dashed line) only coronary flow was recorded. The overflow in the atrial bubble trap, the coronary and aortic' flow were returned to an extra inlet in the middle of the oxygenator

Experimental procedures During the actual experiment, which normally lasted 45–60 min, the hearts worked antegradely i.e. produced an external work. The hearts were throughout paced at 300 beats/min with square wave stimulator at pulse duration of 4 ms and 2–4 V. They were contracting against different levels of peak aortic pressures (50, 80 110 130 and 150 mmHg) where for each level of afterload the preload was varied between 1 and 20 mmHg while CO and coronary flow were measured. On the basis of these measurements Frank-Starling curves, representing different levels of afterload, could be constructed.

Fig 3 illustrates short sequence of an original recording. There was no substantial change in coronary flow

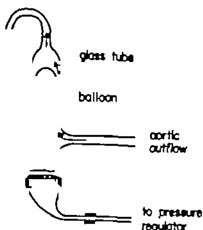


Fig 2 Modified Starling resistor By adjusting the air pressure in the balloon the aortic pressure can be held constant at any desired level

when the preload was successively raised as long as the afterload was kept constant Every 10th min during the recording period CO was checked during standardized conditions i e at preload 5 5 mmHg and afterload 80 mmHg to control that the basal performance and general condition of the heart had not been altered If CO then had decreased more than 10% the experiment was terminated. After the perfusion the wet weight of the left ventricle (including the interventricular wall) was calculated and related to the body weight of the rat

Statistical analyses were performed according to group comparisons Student s *t* test Values are presented as mean ± S E

RESULTS

In the awake SHR ($n=17$) mean arterial pressure (MAP) was 148±1 mmHg at an age representing early phases of established' hypertension while MAP was 100±1 mmHg in the matched NCR ($n=17$) (Table 1) Heart rates were largely the same during the awake state in the two groups It is also seen from Table 1 that the weight of the SHR left ventricle was 35% larger than that of NCR per unit body weight

Fig 4 shows the compiled experimental results concerning the Frank Starling relationship for paced SHR and NCR hearts at 5 different levels of constant afterload ranging from 50 mmHg to 150 mmHg At the lower afterloads (50 mmHg and 80 mmHg) stroke volumes were lower in SHR than in NCR at comparable filling pressures up to 10–1? mmHg where the SHR stroke volume leveled off around 80 µl/100 g b w At an afterload of 50 mmHg the NCR left ventricle reached about the same maximal stroke volumes as SHR already at preloads between 8 and 10 mmHg In NCR stroke volume decreased significantly at still higher preloads ($P<0.05$) which was not the case in the SHR heart even if the filling pressure was here raised to 30 mmHg

The highest stroke volumes obtained during the experimental procedures were 77±3 µl/100 g b w for NCR and 82±2 µl/100 g b w for SHR which for both were obtained at an afterload of 80 mmHg, though SHR needed a higher preload than NCR to reach this maximum i e 15 vs 10 mmHg At increasing afterloads the stroke volumes were more reduced in NCR than in SHR at any given diastolic filling pressure As can be seen from Fig 4 a rise in afterload from 80 mmHg (arbitrarily set to represent the normotensive level) to 130 mmHg (arbitrarily set to represent the hypertensive level) has much a greater influence on the stroke volumes in NCR than in SHR at any given diastolic filling pressure above 5 mmHg The maximal stroke volume/100 g b w is then reduced 30% in NCR but only 11 per cent in SHR (Fig 5) These differences are even more pronounced when afterloads of 80 mmHg and 150 mmHg are compared Thus at a 150 mmHg after load the maximal stroke volume was in SHR reduced by only 21% but by 48% in NCR compared with those obtained at 80 mmHg (Fig. 5) At afterloads of 130 and 150 mmHg the SHR curves

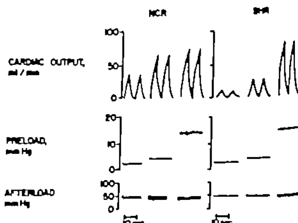


Fig 3 Original registration of cardiac output from one NCR and one SHR heart. The hearts were paced at a frequency of 300 beats/min At constant afterload (50 mmHg in peak aortic pressure) the cardiac output was measured at three different preloads. Note the much smaller output, i e stroke volume of the SHR left ventricle at low preloads while maximal stroke volumes at high preload are the same in SHR and NCR

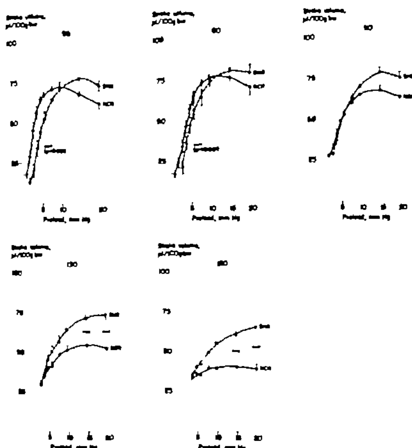


Fig 4 The effect of increased preload on stroke volume per 100 g b.w of adult SHR and age matched NCR left ventricles at 5 different afterloads, ranging from 90 to 150 mmHg Vertical bars represent ±S.E =0 05>P>0.01 0 01>P>0 001 ***=P<0 001

are much steeper than the NCR ones (Fig 4) indicating that the NCR left ventricle is then working near to its maximal performance already at low filling pressures In contrast, the SHR left ventricle has at these high afterloads a far better capacity to regulate its work performance by alterations in preload These findings clearly illustrate that the hypertrophied hearts of SHR can produce a better external work than those of NCR at high afterloads. But at lower afterload the SHR hearts need a higher diastolic filling pressure to produce the same stroke volume as the NCR hearts.

DISCUSSION

A proportionality between the extent of increase in mean arterial pressure (MAP) and left ventricular hypertrophy has been demonstrated both in hypertensive man and in SHR (cf Pickering 1968, Farmer et al 1974) It has however been discussed whether the left ventricular hypertrophy represents a physiologic adaptation (Beznak 1958 Hallbäck et al. 1975 Pfeffer et al 1975) or a degenerative phenomenon (Spann et al 1969 Ross & Sobel 1972, Averill et al 1976) Therefore the functional consequences of the left ventricular hypertrophy were examined in a systematic manner in the present study

The performance of the left ventricle depends on several factors like the enddiastolic filling pressure the inotropic state of the myocardium as governed by neurogenic and hormonal influences and to some extent on the aortic pressure level (afterload) (Sarnoff & Mitchell 1962) When comparing

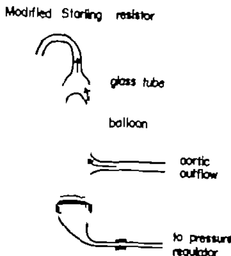


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The highest stroke volumes obtained during the experimental procedures were 77±3 μl/100 g b w for NCR and 82±? μl/100 g b w for SHR which for both were obtained at an afterload of 80 mmHg though SHR needed a higher preload than NCR to reach this maximum i e 15 vs 10 mmHg At in creasing afterloads the stroke volumes were more reduced in NCR than in SHR at any given diastolic filling pressure As can be seen from Fig 4 a rise in afterload from 80 mmHg (arbitrarily set to represen the normotensive level) to 130 mmHg (arbitrarily set to represent the hypertensive level) has much a greater influence on the stroke volumes in NCR than in SHR at any given diastolic filling pressure above 5 *mmHg The maximal stroke volume*/100 g b w is then reduced 30% in NCR but only 11 per cent in SHR (Fig 5) These differences are even *more pronounced when afterloads of 80 mmHg* and 150 mmHg are compared Thus at a 150 mmHg after load the maximal stroke volume was in SHR reduced by only 21% but by 48% in NCR, com pared with those obtained at 80 mmHg (Fig 5) At afterloads of 130 and 150 mmHg the SHR curves

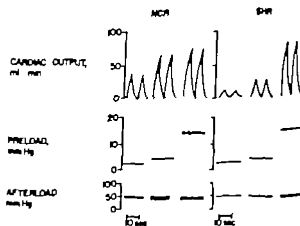


Fig 3 Original registration of cardiac output from one NCR and one SHR heart. The hearts were paced at a frequency of 300 beats/min. *At constant afterload (50 mmHg* in peak aortic pressure) the cardiac output was measured at three different preloads. Note the much smaller output, i.e stroke volume of the SHR left ventricle at low preloads *while maximal stroke volumes at high preload are* the same in SHR and NCR.

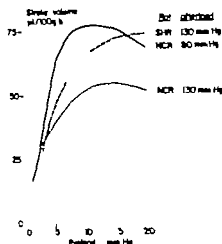


Fig. 6. The effect of increased preload on left ventricular stroke volume per 100 g b.w. for (1) NCR at an afterload of 80 mmHg (corresponding the situation for NCR in vivo), (2) SHR at 130 mmHg (corresponding the situation for the SHR left ventricle in vivo) and (3) NCR at an afterload of 130 mmHg (corresponding the situation in vivo has a normotensive heart is exposed to acute hypertension without the aid of myocardial hypertrophy or if hypertrophy failed to occur in chronic hypertension).

the increased muscle mass of the SHR heart increases its strength proportionally so that it can sustain its stroke volume also when working against high afterload. When comparing stroke volumes in SHR and NCR at given preloads SV is lower in SHR than in NCR at low afterload but higher at high afterload.

However, in the in vivo situation the SHR heart works against a higher afterload than the NCR heart and this situation is mimicked if the Frank Starling relationships obtained in vitro are compared at an afterload of 80 mmHg for NCR and at 130 mmHg for SHR, which largely represents their MAP levels in vivo. In Fig. 6 where this comparison is illustrated the SHR curve is then clearly displaced to the right of the NCR one but when compared to the NCR curve obtained at 130 mmHg afterload, which reflects how a nonhypertrophied heart would work at hypertensive levels the SHR curve is displaced to the left. Fig. 6 thus illustrates that the SHR left ventricle thanks to its hypertrophy better can maintain an adequate stroke volume at the increased afterload, than would have been possible without any structural myocardial adaptation. However when the cardiac function curves are expressed as in Fig. 6, where the SHR and NCR hearts are working against afterloads, which could represent their respective in vivo situation, the SV for a given preload is reduced in SHR compared to that in NCR. This would indicate that if no compensatory adjustments occur stroke volume would tend to decrease along with the progression of hypertension and left ventricular hypertrophy. Several studies also point in this direction both concerning essential hypertension in man (Frohlich et al. 1971) and in SHR (Frohlich & Pfeffer 1973). At least in the early established phases of essential hypertension in man and in SHR stroke volume is however usually within the normal range after being somewhat increased in early more or less labile phases (Pfeffer & Frohlich 1973, Julius & Schork 1971). The present results were obtained in hearts from SHR in the established phase of hypertension when SV does not differ essentially from age matched NCR in vivo (Frohlich & Pfeffer 1973, Noresson, Jones & Hallbäck 1977). When the pumping ability of hearts in established SHR hypertension was evaluated in open chest animals in vivo there were no signs of deteriorated myocardial function (Pfeffer et al. 1976). Therefore some compensation for the altered Frank Starling relationship would probably be at hand in the intact SHR. This could either be in terms of an increased cardiac filling or by an increased inotropic state where the former alternative seems to be the most pronounced mechanism in the SHR (Noresson et al. 1979).

In conclusion. The present results illustrate that the left ventricular hypertrophy has at least two important functional consequences. First, the reduced diastolic compliance of the left ventricle will decrease the stroke volume at low filling pressures, equivalent with a rightward shift of the Frank Starling curve. This is most evident in the lower afterload range. Second, the hypertrophy contributes to make the SHR heart correspondingly stronger so it can better cope with the greater work load that it is exposed to in the hypertensive state. Both these phenomena are consequences of the structural adaptation of the heart to an increased pressure load and are not per se evidence of any myocardial degeneration, though such can, of course, be superimposed in later phases of hypertension. Instead, the absence of hypertrophy in hypertension would lead to reduced cardiac performance with lowered stroke volume and lead to deterioration of cardiovascular homeostasis.

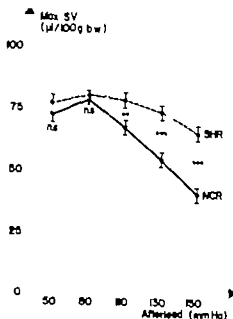


Fig 5 The relation between maximal stroke volume per 100 g b w and afterload for 17 pairs of SHR and NCR left ventricles Vertical bars indicate ±S E ∗=0 05>P>0 01 ∗∗=0 01>P>0 001 ∗∗∗=P<0 001

the performance of hearts with and without hypertrophy all these factors must be kept constant which for practical reasons is almost impossible in vivo (Ross 1976) This comparative study was therefore performed on isolated perfused hearts from SHR and NCR in a system where the abovementioned factors determining cardiac performance could be kept constant or varied independently of each other The impact of changes in enddiastolic filling pressure on stroke volume i e the Frank Starling relationship was earlier investigated in isolated SHR and NCR at afterloads around 60 mmHg (Hallbäck et al 1975) As in the present study a rightward shift in the Frank Starling relationship was then documented of the SHR heart and the extent of this shift seemed to be proportional to the degree of left ventricular hypertrophy However in intact animals the SHR hearts work against a higher afterload than the NCR ones which no doubt affects also the work performance The present results have shown that the work performance of hypertrophied and normal hearts is differently affected by changes in afterload Thus when the SHR and NCR hearts are compared at lower afterloads the Frank Starling relationship for SHR is shifted to the right of that in NCR When however afterload is increased the relationship between filling pressure and SV is better maintained in SHR and at very high afterloads the SHR curve is displaced to the left of that of the NCR at least at higher filling pressures

The rightward shift of the SHR curve at low afterloads could theoretically be explained by a depressed myocardial function This is however an unlikely explanation to judge from the cardiac function curves of SHR and NCR at higher afterloads As earlier discussed by Braunwald & Ross (1969) other factors than depressed myocardial function can be responsible for an altered Frank Starling relationship for example a reduced diastolic compliance Along with hypertension and concomitant left ventricular hypertrophy diastolic compliance is apt to be reduced (Meerson 1969) Then according to Laplace s law the thicker wall of the hypertrophied SHR left ventricle would lead to a correspondingly lower enddiastolic tension *per unit wall thickness* at a given enddiastolic pressure level (cf Hallbäck 1975) The average muscle fibre stretching which determines stroke volume would then be smaller than in the normal more thinwalled left ventricle Thus the more the left ventricular wall lumen ratio is increased by hypertrophy the less pronounced is the myocardial prestretch for a given diastolic filling pressure increase particularly so in the outer wall layers It can hence be concluded that there is an altered relationship between enddiastolic pressure and enddiastolic tension in the presence of an increased myocardial wall thickness [Laplace s law $T=(P \times r)/n$ T=tension P=enddiastolic pressure r=internal radius (unchanged) n=wall thickness (increased)] (Meerson 1969 Hallbäck et al 1975) This disproportion is therefore suggested to cause the shift of the SHR Frank-Starling relationship Such a shift was shown to occur also in renal hypertensive rats by Averill et al (1976) but was here interpreted mainly as signs of degenerative changes

At low afterloads (50 and 80 mmHg) there was no difference between maximal stroke volumes in the SHR and NCR left ventricles This indicates that the rightward shift of the Starling curves in SHR at low afterloads and preloads is not a degenerative process but rather reflects the reduced diastolic compliance Evidently if only the diastolic filling pressure is increased enough as to ensure adequate diastolic prestretch the hypertrophied SHR heart performed just as well as the normotensive one Further at high afterloads (110 130 and 150 mmHg) the SHR hearts produced significantly greater stroke volumes than the NCR hearts (Fig 5) Thus

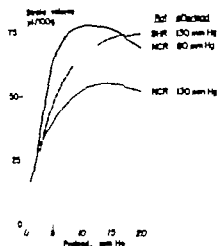


Fig 6 The effect of increased preload on left ventricular stroke volume per 100 g b.w for (1) NCR at an afterload of 80 mmHg (corresponding the situation for NCR in vivo), (2) SHR at 130 mmHg (corresponding the situation for the SHR left ventricle in vivo) and (3) NCR at an afterload of 130 mmHg (corresponding the situation in vivo when a normotensive heart is exposed to acute hypertension without the aid of *myocardial hypertrophy or if* hypertrophy failed to occur in chronic hypertension)

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This investigation was supported by grants from the Swedish Medical Research Council (no 14X-00016 and 14X-04764) the Swedish National Association against Heart and Chest Diseases and the Medical Faculty University of Göteborg

AB Hässle generously covered part of the expenses for a technician

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Left atrial pressure in normotensive and spontaneously hypertensive rats

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NORESSON E RICKSTEN S. E. & THORÉN P Left atrial pressure in normotensive and spontaneously hypertensive rat. Acta Physiol Scand 1979 107 9–12. Received 13 June 1978 ISSN 0001-6772. Department of Physiology University of Göteborg, Sweden.

The left atrial pressure in adult spontaneously hypertensive rats (SHR) of the Okamoto strain and normotensive control rat (NCR) was measured via chronically implanted catheters. In SHR left atrial pressure in end-expiration was more than twice as high (10.3±0 4 mmHg) as in NCR (4.6±0 3 mmHg). There was no difference in the intrapleural pressure between the two groups of rats therefore the enhanced left atrial pressure in SHR represents real rise in the diastolic filling pressure of its left ventricle This is considered to be the most important compensation for the earlier reported rightward shift of the Frank Starling curve in SHR (Hallback Isaksson & Noresson 1975 Noresson et al. 1979 a) Without this compensation the stroke volume would have been drastically reduced for the hypertrophied heart.

Key words Essential hypertension, spontaneously hypertensive rat, left atrial pressure mean arterial pressure

During the development of arterial hypertension in the spontaneously hypertensive rat (SHR, Okamoto 1963) there is a left ventricular hypertrophy largely proportional to the pressure increase (cf Folkow et al 1973) This increased wall thickness of the left ventricle leads to a decreased compliance shifting the Frank Starling curve to the right for SHR compared to NCR (normotensive control rat) (Hallback, Isaksson & Noresson 1975) To compensate for this Frank Starling shift, the heart could either work on higher inotropic state i e shift to another steeper Starling curve or climb upwards on the prevailing curve by increasing the diastolic filling pressure of the left ventricle or a combination of both. To explore which of these alternatives is the prevalent one in SHR in vivo the left atrial pressures were measured via chronically implanted catheters at rest in awake NCR and SHR.

Further recent data by Thorén et al (1979 a, b) indicate that the left atrial receptors are reset in SHR. Data on left atrial pressures in NCR and SHR will help to determine the degree of activity in vivo in these receptors.

METHODS

4–5 months old male SHR (weighing around 340 g) and NCR of the same age and sex but with moderately higher body weight (around 420 g, Table I) were used. Seven technically successful expts. were obtained in each strain of rats out of a total of about 40 operated rats. The rats had water ad. libitum and were fed on a standard pellet diet. During brief ether anesthesia PE 50 cannula was inserted into the tail artery The animals were allowed to wake up and the awake mean arterial pressure (MAP) and heart rate were recorded on Grass polygraph (model 7). Five SHR with MAP below 135 mmHg and one NCR with pressure higher than 110 mmHg were excluded from the study

After these measurements the animals were anesthetized with sodium pentobarbital (Nembutal®) 40–50 mg/kg b w i.a. and 1/4 of the dose was given when needed. The rats were intubated orally with PE 200 tube and artificially ventilated with positive pressure system (max. inspiration pressure 15 cm water). The thorax was opened in the 4th intercostal space on the right side and great care was taken not to damage the lungs, the intercostal artery or the internal mammary artery

Two hemostats were placed on the lateral parts of the right middle lung lobe in order to expose the pulmonary vein The vessel was ligated off distally and suture was placed around the base of the lobe Under dissection-

Table 1 *Blood pressure heart rate body weight and left atrial pressure (at the end of the expiratory phase and the mean value) in adult SHR and matched NCR*

Values presented are mean ± S E Levels of significance are given

	Blood pressure (mmHg)	Heart rate (beats/min)	Body weight (g)	Left atrial pressure (mmHg)	
				In end-expiration	Mean
SHR (n =7)	140±2	368±7	340±2	10.3±0 4	6 8±0.3
NCR (n =7)	102±2	379±5	422±4	4 6±0 3	2_±0.4
$P<$	0 001	n s.	0.001	0 001	0 01
SHR/NCR	1.39	0.97	0.81	2.24	3 09

microscope a 1.5–2 cm long PE 10 tube connected to a PE 50 catheter was inserted into the lung vein By manipulating the snare bleedings could be kept minimal The tip of the catheter was placed into the left atrium and was connected to a pressure transducer (Statham 23 DC) writing on the Grass polygraph This catheter passed through the muscle layer in the 5th interstitium and another PE 50 catheter was introduced in the same interstitium with the tip in the thoracic cavity 1 mm inside the wall Air and exudate could be sucked through the pleural catheter during the closure of the thorax and after the operation

When closing the thorax the animal lungs were blown up to a respiratory pressure of 20–25 cm water the respiration switched off and the sutures tighted, whereupon respiration was started again. The two catheters were secured into the thorax wall and then subcutaneously led up to the neck and exteriorized The catheters were filled with heparinized saline. The muscle and skin layers were sutured with single sutures The suture lines were more than 1 cm away from the 4th intercostal space in order to prevent leakage of air During the whole operation the left atrial pressure was followed to ensure good communication. Later the animal was taken off the respirator and subsequently extubated Body temperature was followed during and after the operation and held at 37°C–38°C After the operation the remaining air in the thoracic cavity was sucked out. It was important to repeatedly suck the trachea during the postoperative period to avoid respiratory failure Acute postoperative complications were arterial embolies (hindlimb kidney) and pneumothorax with a total mortality of about 35%

After a postoperative rehabilitation period of 7 days in individual cages the left atrial pressure was measured The two catheters were connected to pressure transducers (Statham 23 DC) placed 30 mm above the cage floor Thus the zero value for the pressures was 30 mm above the floor where the left atrium was estimated to be when the rat was in resting position During 30 min the rats were allowed to rest in the cage great care being taken to maintain a quiet environment Both damped and undamped left atrial pressures and in 4 rats also pleural pressures were measured when the rats were in basal conditions. In 8 operated rats the communication in the left atrial catheter was unreliable and these rats were excluded The pre- and postoperative MAP levels did not differ by more than 10 mmHg

RESULTS

MAP levels after light ether anesthesia were 140± mmHg in SHR and 101±? mmHg in NCR (Table 1) Table 1 also shows heart rate body weight, left atrial pressure at the end of the expiratory phase together with mean left atrial pressure all recorded during rest for the two groups of rats. SHR had then slightly lower heart rates than NCR as earlier reported by Noresson Jones & Hallbäck (1977) Left atrial pressure at the end of expiratory phase was 124% higher in SHR than in NCR (10.3±0 4 mmHg and 4 6±0 3 mmHg, respectively $P<0\ 001$ Table 1) The mean value for left atrial pressure was 209% higher for SHR compared to NCR (6.8±0 3 mmHg and 2.2±0 4 mmHg respectively $P<0.01$, Table 1)

Fig 1 shows the relation between MAP and left atrial pressure in the end of the expiratory phase for SHR and NCR There is a clear separation between the two groups with the highest atrial pressure at 6 mmHg for NCR and the lowest for SHR at 6 5 mmHg. In two SHR and two NCR the intrapleural pressures at the end of the expiratory phase were the same and varied between 0 and ? mmHg indicating that there really is a higher filling pressure of left ventricle in SHR than in NCR In the other experiments the pleural pressure measurements failed because of the great tendency of fibrin formation around the tip of the pleural catheter

DISCUSSION

In the present study the left atrial pressure was measured via a thin chronically implanted PE 10 catheter in awake adult spontaneously hypertensive rats (SHR Okamoto 1969) and normotensive control rats (NCR) SHR is considered to be a good



Fig 1 Relationship between mean arterial blood pressure (mmHg) and left atrial pressure in end-expiration (mmHg) for adult SHR (●) and matched normotensive controls (NCR ○) Vertical and horizontal bars represent ± S E Note that there is no overlapping between the two groups

animal analogue for essential hypertension in man. The peak left atrial pressure value at the end of expiration averaged 10.3±0 4 mmHg for SHR and 4 6±0.3 mmHg for NCR These figures are 0.5 to 1 0 mmHg lower if instead the mean pressure at end expiration is registered because the left atrial pulse amplitude measured via the PE 10 tube was 1–2 mmHg at the end of the expiratory phase. Every inspiration was recorded as a shortlasting sharp dip (about 5 mmHg) in the pressure registration, with no differences between SHR and NCR If the left atrial pressure tracing curve is damped the pressures will be further reduced to 6.8±0.3 mmHg for SHR and 2..±0 4 mmHg for NCR because of the influence of the low pressure during the inspiratory period. As there is no difference in the intrapleural pressure between the two groups of rats, these higher values for SHR represent greater transmural pressure in the left atrium and an enhanced filling pressure for the hypertrophied left ventricle

There could be at least two reasons for the increased pressure in the left atrium and the pulmonary veins in the hypertensive animals First, there may be a centralization of blood to the cardiopulmonary area, as reported in the labile form of essential hypertension in man (Julius & Esler 1976) Second, the elevated left atrial pressure could be caused by an increased stiffness of the lung veins and/or of the left atrium. Such a reduced distensibility of these walls could be caused by an increased discharge in the sympathetic nerves to the cardiopulmonary area and/or by a secondary wall hypertrophy caused by the enhanced pressure (cf Folkow et al 1973)

The functional importance of such an increased filling pressure of left ventricle is obvious. As earlier reported the increased thickness of the SHR left ventricular wall reduced its compliance, which causes a shift of the Frank Starling relationship to the right (Hallbäck Isaksson & Noresson 1975) This displacement is probably of great importance for the gradual reduction of cardiac output, when the hyperkinetic initiating form of essential hypertension is transverted into the more fixed form with normal or somewhat lowered cardiac output (Lund-Johansen 1978) The raised left ventricular filling pressure is thus of great importance as a compensation for this Frank Starling shift, helping the heart to preserve its stroke volume in the hypertrophied state Without this compensation the stroke volume and hence cardiac output would be drastically reduced The other possible compensatory mechanism for the displaced Starling curve is an enhanced ventricular inotrophy but data from our laboratory indicate that this is of relatively minor importance in SHR (Noresson et al 1979 b)

Unmyelinated cardiac receptor endings especially those on the left side are of importance for the regulation of blood volume (Thorén et al 1976 cf. Thorén 1979). Rats have a large population of cardiac receptors in the atria and all of them seem to transmit their information via unmyelinated fibres (Thorén et al. 1979 a) The threshold of these receptors is clearly reset to a higher level in SHR with established hypertension (Thorén et al 1979 b) compared to NCR (Thorén et al 1979 a) probably because of secondary structural changes in the atrial wall

To summarize Parallel with the MAP rise in SHR cardiac hypertrophy occurs which makes the left ventricular wall thicker and less distensible leading to a right ward shift of the Frank Starling curve This would tend to lower the stroke volume and reduce cardiac output. As a secondary compensatory mechanism there is a rise in left atrial pressure to maintain an adequate filling of the left ventricle The precise mechanisms behind this left atrial pressure rise are not known

This work was supported by grants from the Swedish Medical Research Council (14X-00016 and 14X-04764) from the Faculty of Medicine University of Göteborg and from Svenska Sällskapet for Medicinsk Forskning AB Hässle Göteborg has generously covered part of the expenses for a technician Thanks to Carl Widmark for skilful technical assistance

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THORÉN P NORESSON E. & RICKSTEN S -E.. Resetting of cardiac C-fiber endings in the spontaneously hypertensive rat Acta Physiol Scand 1979 107 13–18. Received 13 June 1978. ISSN 0001-6772. Department of Physiology University of Göteborg Sweden.

The characteristics of 11 left atrial receptors in 9 adult male spontaneously hypertensive rats (SHR) were investigated. All the receptor afferents were non-medullated with conduction velocities from 0.5 to 1.5 m/s. Elevation of left atrial pressure during graded aortic occlusion always induced marked increase in receptor discharge with maximal frequencies ranging from 29 to 70 Hz. The threshold for activation was from 7.5 to 13 mmHg in mean left atrial pressure (mean ± S E. 10._±0.6 mmHg). Upon atrial distension all the receptors displayed clear rhythmicity and the discharge correlated mainly with the v-wave indicating distension as the cause of receptor activation. The relationship between the mean left atrial pressure and the frequency of discharge was constructed for all the receptors and compared with similar data obtained from normotensive control rats (NCR) (Thorén et al. 1979) It was then obvious that the left atrial receptors are reset in SHR probably secondarily to decreased distensibility of the left atrium.

Key ords. Primary hypertension, spontaneously hypertensive rat, volume receptors, C-fiber endings, atrial pressure

In dogs and cats there is a population of atrial receptors with medullated vagal afferents situated mainly at the ein-atrial junctions (Paintal 1973) More recently atrial C-fiber endings in the heart of dogs and cats have also been described (Coleridge et al 1973, Thoren 1976) In the normotensive rat (NCR) heart Thorén et al (1978) found a large number of afferent C-fiber endings in the atria, but they ere not able to find any atrial medullated afferents or any afferents from the ventricles. Most of the atrial C-fiber endings had very high firing frequencies with cardiac rhythmicity up to 50–60 Hz, but some of the receptors showed a more low frequent irregular type of discharge with maximal frequencies les than 25 Hz.

Resetting of cardiac receptors has been shown to occur during chronic severe heart failure n dogs (Greenberg et al 1973) In their preparation there was a marked derangement of the receptor function, probably due to a severe degeneration of the receptor field and to an altered distensibility of the atrium (Zucker et al. 1977)

The aim of the present study was to examine the characteristics of these left atrial C-fiber endings in the spontaneously hypertensive rat of the Okamoto-strain (SHR) and to compare these characteristics with similar data earlier obtained from the normotensive control rat (NCR) (Thorén et al 1979). This study provides for the first time evidence for a resetting of cardiac receptors in a hypertensive animal.

METHODS

Experiments were performed on 9 male spontaneously hypertensive rats (SHR) of the Okamoto strain (300–380 g, age 12– 4 weeks). Under light ether anesthesia PE 50 catheter was inserted into the tail artery The mean arterial pressure (MAP) was recorded on Grass polygraph when the animal was awake after ending the anesthesia. The animals were then anesthetized with sodium pentobarbital (Nembutal® 40–50 mg/kg) administrated i.a. and the anesthesia was maintained by repeated doses (Nembutal® 10–15 mg/kg, every 60 min). The trachea was cannulated with a PE-200 tube and positive pressure ventilation with pure oxygen was started with frequency of 25 per minute and an end-expiratory pressure of 12–15 cm water All details of the preparation have been published previously (Thorén et al. 1979) and the method will only be described in short here. Muscle movements were prevented by gallamine (Flaxedil® 2–4 mg/kg i. ., repeated every 60 min).

Surgical procedure The right vagus nerve and the

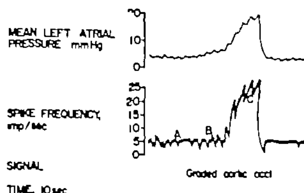


Fig 1 Mean left atrial pressure and *spike frequency in a* single left atrial C-fiber during a graded elevation of left atrial pressure induced by a graded aortic occlusion

carotid artery were dissected free in the neck The thorax was opened by a bilateral intercostal transsternal incision Snares were placed around the ascending aorta and the inferior caval vein A thin catheter (PE 10) was placed in the left atrium through the right middle lung lobe vein and connected to a low volume displacement transducer (Gaeltic 3EA) The right upper and middle lung lobe were removed Arterial blood pressure was recorded *in the tail artery via a Statham 23DC transducer* The right jugular vein was cannulated The vagal trunc was identified just above the lung root and cut in order to eliminate most of the vagal afferents from the lungs and abdominal viscera

Recordings The right cervical vagus was placed on a dissection plate and the nerve sheath was carefully dissected away by means of sharp forceps under a dissection *microscope Thin filaments were then obtained and cut* centrally and placed on a thin bipolar silver electrode and connected to a nerve amplifier (Grass p 511) The output of the amplifier was connected to a loudspeaker a rate meter an oscilloscope and to an UV-recorder (ABEM 5651) writing intermittently with fast speed Blood pressure mean left atrial pressure and the output of the rate meter were recorded on a Gras (mod 7) recorder writing *continuously with slow speed The undamped left atrial* pressure and ECG were recorded on the UV-recorder together with the neurogram (Fig 2)

Experimental procedures A a screening procedure to find receptors in the left side of the heart the activity in all dissected filament were observed during short lasting (3–6 s) occlusion of ascending aorta. Every filament that responded with increased activity to this manoeuvre was dissected further until a filament with only one active fiber was found In most instances an exact localization of the receptors wa obta ned by probing the heart with a fine probe *after killing the animal and open*ing the heart The total conduction time was obtained by placing a stimulation electrode connected to a Grass (S4) stimulator over the receptor site and record ng the evoked potential in the neck The conduction velocity was estimated from the approximate length of the conduction pathway *and the total conduction time*

Receptor activity was recorded during control conditions *and during graded* elev t on of left atrial pressure (*graded aortic occlusion*) The receptor characteristics were compared with the earlier obtained data from the NCR and significance was determined by Student *t*-test Values are presented as mean ± S.E

RESULTS

This study is based on recording from 11 left atrial receptors in 9 spontaneously hypertensive rats (SHR) The MAP of the awake rats were 151 ± mmHg. Total conduction time from the receptor site to the recording electrode was from 20–61 ms and the conduction velocity varied from 0.5 to 1.5 m/s (0 8±0 1 m/s) All the receptors were located in the left atrium as evidenced by probing with a fine plastic probe Six of the receptors were localized by probing the opened non-beating heart and the other 5 were localized by probing the intact beating heart The *studied receptors were located throughout the* entire left atrium also in the atrial appendices and the first part of pulmonary veins

Receptor discharge during elevation of left atrial pressure All the receptors responded to elevation of the left atrial pressure induced by a brief occlusion of ascending aorta and their maximal frequency varied from 29 to 70 Hz (55±3 Hz) The response to a graded increase of the left atrial pressure is shown in Fig 1 The corresponding neurograms are shown in Fig 2 Notice that this receptor had a low *frequency spontaneou discharge* with 1 impulse per cardiac cycle and that upon aortic occlusion the *activity increased in parallel* with the elevated left atrial pressure

Fig 3 shows the individual pressure-response curves for all the 11 receptors Notice the marked variation in the pressure response curves The threshold upon activation of the individual receptors varied from 7 5 to 13 mmHg (10.–±0.6 mmHg) in mean *atrial* pressure

The averaged data relating mean left atrial pres*sure and the activity in the receptors* i shown in Fig 4 A

Firing pattern f the recept rs In the control situation 7 receptors showed a cardiac modulated discharge and when taking the total conduction time into account 4 of them were activated during the a wave Upon elevation of left atrial pressure (aortic occlusion) *the activity in all the receptors* showed a clear cardiac rhythmic discharge pattern and the firing wa correlated with the v wave However some endings *also discharged with 1 or 2* spikes every a-wave during high atrial pres ures

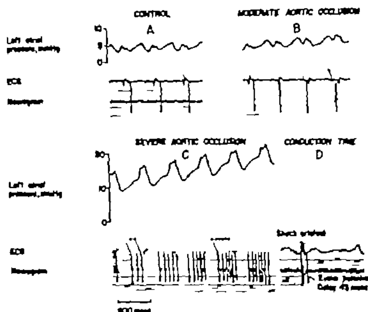


Fig. 2. Left atrial pressure ECG and nerve impulses in left atrial C-fiber during control conditions (A), moderate aortic occlusion (B), and severe aortic occlusion (C). This receptor is the same as shown in Fig. 1 A. In the control condition the receptor is activated during the a-wave as indicated by the asterisks showing the position in the cardiac cycle of the receptor activation after correction for the total conduction time from the receptor site to the recording electrode as shown in D. Upon a moderate elevation of atrial pressure (B) the firing occurs during the -wave and upon further elevation of the left atrial pressure the v-wave discharge is more prominent with up to 7 imp every -wave.

Comparison of left atrial receptor discharge in NCR and SHR. In Fig. 4 A the mean discharge from 13 atrial receptors in NCR (Thorén et al. 1979) and 11 in SHR is compared at increasing mean left atrial pressures. All the receptors in the normotensive rats responded to pressures below 10 mmHg. The thresholds of the SHR receptors were most often above 10 mmHg. Thus, a clear resetting of the atrial C fiber endings in the hypertensive rat is evident.

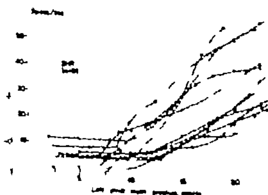


Fig. 3. The pressure-response curves for 11 different left atrial receptors in 9 rats. The activity in the individual receptors is plotted against left atrial mean pressure during an elevation of left atrial pressure induced by a graded aortic occlusion.

It has been suggested by Thorén et al. (1979) that there are populations of atrial C-fiber endings in the heart of the NCR. One group of endings has a low frequent irregular discharge and the other type has a more frequent irregular type of discharge. All of the receptors described in the present study were of the high frequency type with cardiac rhythmicity upon activation and maximal frequencies above 25 Hz. If the left atrial receptors in SHR are compared with the earlier obtained data on high frequency receptors in the normotensive animals, the resetting of the left atrial receptors in SHR is still more evident (Fig. 4 B).

DISCUSSION

The presence of cardiac vagal receptors has been known since many years. Thus, Paintal (1973) de-

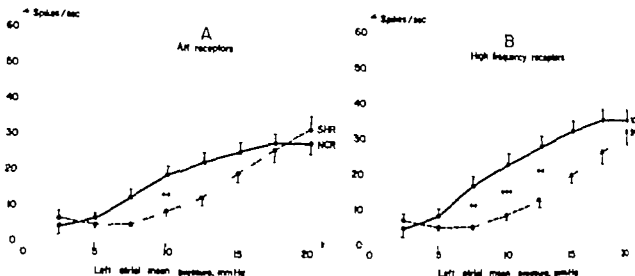


Fig 4A Activity in non-medullated vagal afferents from the left atrium during increases in left atrial pressure caused by aortic occlusion The interrupted line shows mean ± S E of recordings from 11 single fibers For comparison earlier obtained data from NCR are also shown (solid line) (means $P<0.05$ means $P<0.01$).
Fig 4B The mean activity in 11 left atrial high frequency C-fiber endings in SHR (interrupted line) and similar obtained data from 8 high frequency receptors in NCR (solid line) The data are plotted in the same way as in Fig. 3

scribed atrial receptors with medullated afferents mainly located at the vein-atrial junctions More recently Coleridge et al (1973) and Thorén (1976) examined the characteristics of atrial receptors with *non-medullated vagal afferents in dogs and cats* These receptors had somewhat higher thresholds and lower discharge frequencies but many of the atrial C-fiber endings could also respond to moderate elevations of left atrial pressure There is also a large population of nerve endings with non-medullated vagal afferents *in the left ventricle* (Coleridge et al 1964 Sleight & Widdicombe 1965 Öberg & Thorén 1972 Thorén 1977) This group of cardiac C-fiber endings seemed to have a tonic influence on vasomotor center (Thorén et al 1976)

Thorén et al (1979) recently suggested the existence of two populations of left atrial C-fiber receptors in the rat One type of endings showed a fairly low frequent irregular discharge upon elevation of left atrial pressure while the other type was activated with a more high frequent cardiac modulated discharge The low frequent irregular receptors had higher thresholds than the high frequency ones

Resetting of cardiac receptors in hypertension In the present examination the characteristics of such C-fiber endings in the spontaneously hypertensive rat (SHR) have been studied and compared to those in NCR In this study only high frequency receptors were found but they had a much higher threshold than the receptors had in the normotensive animals (10 ?±0 6 mmHg for SHR in comparison with 4 6±0 6 mmHg for NCR) It is possible that the low frequency receptors in SHR had very high *thresholds and were thus difficult to find.*

Recent studies by Noresson et al (1979) indicate that there is a marked elevation of left atrial pressure in the hypertensive rats A cellular hypertrophy is a general phenomenon in hypertensive animals both in the left ventricle and in the resistance vessels *This is to a great deal* considered to be induced by the increased tension per individual cell (Meerson 1969 Folkow et al 1973) It is therefore likely that this elevated left atrial pressure will induce hypertrophy of the atrial wall which will cause a decreased distensibility of the wall. The decreased distensibility of left atrium will then probably be the main reason for the resetting of the receptors

Physiological consequences of the resetting of the atrial receptors In cats and dogs the cardiac C-fiber endings induce a tonic vasomotoric inhibition on the vasomotor center and this tonic inhibition seemed to be specifically marked on the vasomotor neurons controlling the sympathetic outflow to the kidney (cf Thorén et al 1976) It has also been postulated that these cardiac vagal reflexes are of importance for the blood volume regulation due to the marked effect on the renal sympathetic outflow Changes in the renal

mpathetic outflow can influence the sodium ex rtion via a direct effect on the tubular cells (Di- na 1977) and via changes in renin release anchetti et al 1976). It was recently shown by ickstein et al (1979) that the tonic renal nerve hibition from cardiac receptors is altered in ypertensive rats. These authors examined the in- bition on the renal nerve upon volume load and lowed that higher left atrial pressures were needed induce the renal nerve inhibition. This further ipports the concept that cardiac receptors are re- t in spontaneous hypertension, and the altered al sympathetic outflow is probably of im- ortance for the reflex control of sodium excretion nd renin release

The resetting of the left atrial C-fibers in the hypertensive rat has several interesting functional consequences. Thus, it has been shown (Hallback t al. 1975) that hypertrophy of the left ventricle oduces a clear shift of the Frank Starling curve to he right meaning that the hypertensive hearts need considerably higher filling pressure to obtain the same stroke volume. As discussed above SHR has a considerably higher left atrial pressure than the normotensive animals. Such a high left atrial pres- sure would in the absence of left atrial receptor resetting induce a marked inhibition of the sympathetic outflow to the capacitance vessels and it ould then be difficult for SHR to ensure an adequate left ventricular filling pressure

Another interesting point is that the renin release in the spontaneously hypertensive rat seemed to be inhibited (Shiono & Sokabe 1977) The depression seemed to be more pronounced in the very young (5 weeks) hypertensive animals when compared with the older animal with more established hyperten- sion despite the marked increase in left atrial pres sure in the older rats. A possible reason might be a resetting of the cardiac C-fibers in established hypertension

Does the resetting of the cardiac receptors in established hypertension n rats have any signifi- cance for pathophysiology of essential hypertension in man There are som recent studies which might indicate this Thus patient with mild essential hypertension and low renin values have on average 30% increase in central blood volume without any change in the total blood volume (Julius & Esler 1976) The increased central blood volume is likely to activate the cardiac receptors and cause the re- flex inhibition of renin release Interestingly enough patients with established and more severe hypertension do not show this inverse relationship between the central blood volume and the renin levels (London et al 1977) One reason might be a resetting of the cardiac receptors in established hypertension n man.

2 Years

This study was supported by S edish Medical Research Council grants 14X-00016 and 14X-04764

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…he effect of different diets and of insulin on the hormonal …response to prolonged exercise

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GALBO H HOLST J J & CHRISTENSEN N J The effect of different diets and of insulin on the hormonal response to prolonged exercise. Acta Physiol Scand 1979 107 19-32 Received 12 Dec. 1978. ISSN 0001-6772. Department of Medical Physiology B University of Copenhagen, Department of Clinical Chemistry Bispebjerg Hospital and 2nd Clinic of Internal Medicine Aarhus Kommunehospital Denmark.

The importance of carbohydrate availability during exercise for metabolism and plasma hormone levels was studied Seven healthy men ran on treadmill at 70% of individual maximal oxygen uptake having eaten diet low (F) or high (CH) in carbohydrate through 4 days. At exhaustion the subjects were encouraged to continue to run while glucose infusion increased plasma glucose to preexercise levels. Forearm venous blood biopsies from vastus muscle and expiratory gas were analyzed Time to exhaustion was longer in CH- (106±5 min (S E.)) than in F-expts. (64±6). During exercise, overall carbohydrate combustion rate, muscular glycogen depletion and glucose and lactate concentrations, carbohydrate metabolites in plasma, and estimated rate of hepatic glucose production were higher fat metabolites lower and the decrease in plasma glucose slower in CH than in F-expts. Plasma norepinephrine increased and insulin decreased similarly in CH- and F-expts whereas the increase in glucagon, epinephrine growth hormone and cortisol was enhanced in F-expts. Glucose infusion eliminated hypoglycemic symptoms but did not substantially increase performance time During the infusion epinephrine decreased markedly and glucagon even to preexercise levels Infusion of insulin (to 436% of preexercise concentration) in addition to glucose in F-expts. did not change the plasma levels of the other hormones more than infusion of glucose only but reduced fat metabolites in plasma. At exhaustion muscular glycogen depletion was slow and the glucose gradient between plasma and sarcoplasma as well as the muscular glucose 6-phosphate concentration had decreased. Conclusions. The preceding diet modifies the energy depots, the state of which (as regards size receptors and enzymes) is of prime importance for metabolism during prolonged exercise Plentiful carbohydrate stores favor both glucose oxidation and lactate production During exercise norepinephrine increases and insulin decreases independent of plasma glucose changes whereas receptors sensitive to glucose privation but not to acute changes in insulin levels enhance the exercise-induced secretion of glucagon epinephrine growth hormone and cortisol Abolition of cerebral hypoglycemia does not inevitably increase performance time because elimination of the hypoglycemia may not abolish muscular energy lack

Key words Glucagon, growth hormone, cortisol norepinephrine epinephrine glucose glycogen, lipid mobilization, metabolism, fatigue hypoglycemia

During prolonged heavy exercise large increases in the uptake and combustion of glucose and free fatty acids occur in the working muscles. At the same time marked changes take place in the plasma concentrations of various hormones changes which favor glucose production and lipid mobilization. The concentration of insulin decreases, whereas the concentrations of catecholamines glucagon, growth hormone and cortisol increase (Galbo et al 1977*c*) We have previously investigated the mechanisms which give rise to these hormonal changes Our studies led to the view that in exercising man plasma norepinephrine mainly reflects a work load dependent activity in the sympathetic nervous system and that α-adrenergic activity inhibits insulin secretion. Furthermore the

hypothesis was put forward that in man during prolonged exercise the secretion of the other hormones is stimulated by cells in CNS and pancreas which respond to glucose privation (Galbo et al 1977*c*)

In order to examine the validity of this hypothesis we have now measured plasma hormone levels during prolonged exercise which was performed after intake through 4 days of a diet rich in either fat or carbohydrate Such regimens have been used several times in Scandinavia in this century in studies of exercise metabolism (Boje 1935 Christensen & Hansen 1939 Bergström et al 1967 Hultman 1967 Hultman & Nilsson 1971) They result in small and large glycogen stores respectively and during exercise the plasma glucose concentration declines more rapidly after a fat diet than after a carbohydrate diet Accordingly if the hormonal response to exercise is enhanced by receptors sensitive to glucose privation also the hormonal changes during exercise would be expected to take place more rapidly after a fat diet than after a carbohydrate diet In order to further clarify the relationship between the hormonal response and changes in glucose concentrations plasma glucose was at exhaustion restored to pre-exercise levels by glucose infusion during continued exercise

A decrease in the tissue concentration of insulin may aggravate intracellular glucose privation in glucose sensitive cells regulating hormonal secretion (Müller Faloona & Unger 1971 Raskin Fujita & Unger 1975 Szabo & Szabo 197?) Accordingly the decrease of insulin concentration in plasma which takes place during prolonged exercise may enhance hormonal secretion during exercise If so simultaneous infusion of insulin and glucose may be more efficient in depressing the hormonal response to exercise than infusion of glucose only Consequently in order to elucidate the importance of insulin for the hormonal response to exercise we infused insulin together with glucose at exhaustion in a group of expts with fat diet Finally to substantiate the influence of the diets on the glycogen depots and to study the effect of different diets on the metabolic state of working muscles biopsies were taken from the vastus muscle

METHODS

Subjects and procedure Seven healthy male students (26 (24–29) years (mean and range)) familiar with the laboratory gave their informed consent to participate in the study The students mean maximal O_2 uptake (V_{O_2max}) determined during treadmill running was 4.33 (3.76–5.1) l min and 56 (52–63) ml kg min Mean weight 77 (67–85.5) kg and mean height 184 (180–189) cm Each subject underwent three diet periods These periods lasted 4 days each and were separated by 17 days during which no restrictions concerning physical activity and choice of food were ordered At the start of each diet period the subjects arrived in the laboratory after an overnight fast After 30 min rest in a chair they were weighed and in the standing position blood drawn without stasis from a forearm vein Then in order to produce glycogen depletion the subjects exercised 45 min at a work load corresponding to 80 % of individual V_{O_2max} 30 min on a treadmill and 15 min on a bicycle ergometer Subsequently a liquid diet (1 000 kcal l) consisting in the first (F_I-expts in the following designated) and third (F_{II}-expts designated —) diet period of 76 % fat and 13.5 % protein, and in the second diet period (*CH-expts* designated. —) of 7? carbohydrate and 13.5 % protein was delivered The subjects were asked to consume an amount of diet corresponding to their estimated energy consumption in order to avoid a negative balance of energy in the diet period The consumed amount was determined by weighing Besides the diet only intake of water and vitamin pills were allowed In the diet periods physical training and smoking were not allowed In the intervals between the diet periods a supplement of iron (Fe 40 mg × day) was given Each diet period was finished by an overnight fast (10 h) after which the students arrived in the laboratory at 8 a.m They were weighed and had a cannula inserted intravenously in the left forearm whereupon they rested in a chair for 30 min A sample of urine was examined for ketone bodies glucose and albumin by stix (Ames) The electrocardiogram was registered with precordial electrodes While the subjects were standing their oxygen uptake at rest was measured with the Douglas bag method and venous blood samples were drawn without stasis. The glucose concentration in capillary blood from a fingertip was determined at once with a reflectance meter (Ames) (Schersten et al 1974) Exercise was performed on a motordriven treadmill with 3 % inclination The speed of the treadmill was chosen so that the work load required 70 % of individual maximal oxygen uptake The subjects ran for repeated 30 min bouts separated by 10-min rest intervals This sequence was continued until exhaustion At exhaustion (Exhaustion I) a 10 min rest was allowed while a cannula was inserted intravenously in the right forearm and connected to a motor-driven infusion pump Then the subjects were encouraged to resume the running while a 25 % (g (100 ml)) glucose solution adjusted to pH 7 with 1 M $NaHCO_3$, was infused from a calibrated glass syringe Every second min the glucose concentration in capillary blood from a fingertip was determined with the reflectance meter and the speed of the infusion pump adjusted to achieve the pre-exercise blood glucose level and to maintain this level until the subjects were unable to run longer (Exhaustion) In the third exercise test (F_{II}-expts) human insulin (Actrapid Novo) was infused as well (1.15 ml min of a solution of 121.8 nmol l of insulin in isotonic sodium chloride containing in addition 1 % (g

70 ml⁻) human serum albumin) after priming-dose 1675 ml kg bw ¹) Expired air was collected through 1.5 min after 20 min of each exercise period 1 each exercise period blood samples were drawn from the cathe ... after 15 min for analysis of glucose, insulin and glucagon and during the last 2 min for analysis of glucagon, insulin growth hormone (GH), cortisol, catecholamines glucose glycerol FFA (free fatty acids), β-hydroxybutyrate alanine lactate pyruvate and haematocrit. Capillary blood from fingertips was sampled at the end of each exercise period for glucose determination. Finally blood samples and expired air were collected ... min after ... the subjects having recovered for 5 min in the supine and for 5 min in the standing position ... mineral water was offered ad libitum 1 the first (F_1) as well as in the third (F_3) experimental series on an average 70 ml of blood were drawn and in the second (CH) 230 ml were drawn. The blood was immediately replaced by isotonic sodium chloride. In the first and second exercise test needle biopsies (Bergström 1975) weighing about 30 mg were obtained from the lateral head of the quadriceps femoris muscle immediately before exercise and immediately after the first and second run and at exhaustion. The biopsies were obtained through 0.5 cm incisions in the skin 12 16 cm above the knee. The incisions were made in one leg in the first exercise test and in the other leg in the second exercise test.

Analyses. The volume of the collected expired air was measured with 150 l gasometer (Collins). The expired air was analysed with an infrared CO_2-analyser (Beckman LB-1 OA 184) and paramagnetic O_2 analyser (Servomex). The accuracy of the analyses was verified with the Scholander microtechnique (deviation less than 0.06 % CO_2 and O_2, respectively at 12 comparisons performed with intervals of 14 days). Blood for hormone determinations was drawn in iced test tubes and centrifuged in the cold. Plasma or serum was stored at −20°C until analysis. For catecholamine analysis 10 ml blood were sampled in test tubes, which contained 70 mg EDTA and 20 mg ascorbic acid. Plasma norepinephrine and plasma epinephrine were determined by double-isotope derivative assay (Engelman & Portnoy 1970) with certain modifications (Christensen 1973). For glucagon and insulin determinations 5 ml blood were drawn in heparinized test tubes containing ... inhibitor 0.25 ml Trasylol (Bayer), 10000 kallikrein-inhibiting units ml⁻ Glucagon was measured with radio-immunoassay after ethanol extraction of plasma (Heding 1971). The antiserum employed 4317 (Holst & Aasted 1974) does not cross-react with enteroglucagon. Purified porcine glucagon (NOVO) was used as standard and bound and free hormone were separated by ethanol. Detection limit was below 5.7 pmol l and intra- and inter-assay coefficient of variation were about 5 % and 15 % respectively. Insulin was determined by radioimmunoassay relying on charcoal separation (Albano et al 1972). Human insulin (NOVO) was used as standard. The detection limit for the assay system was 0.3 pmol l and the intra-assay coefficient of variation (n 20) was 3 % at 46 pmol l (7 pmol l of insulin equal 1 μU ml). Measurements of glucagon and insulin concentration in plasma with added exogenous hormone in dilutions of plasma, and in mixtures of plasma with different concentrations yielded results, which deviated less than 10 % from the expected values. Serum growth hormone (GH) was determined by a single antibody radioimmunoassay employing Wick chromatography (Ørskov Thomsen & Yde 1968). A Wilhelmi preparation (HS 968 C) was used as standard. Serum cortisol was measured by commercially available solid phase radioimmunoassay-kit (CEA-IRE SORIN). The intra- and interassay coefficients of variation of the cortisol assay (=8 triplicate determinations) were 13 % (at 606 nmol l ¹) and 15 % respectively. Serum from adrenalectomized rats yielded values which were not significantly different from zero and recovery experiments yielded results which deviated less than 15 % from the expected values. Every subject had all analyses of hormone carried out in single assay run.

The concentration of glucose in blood and plasma was determined by the glucose-oxidase method (Werner Rey & Wielinger 1970). In respect to glucose concentrations arterialized capillary blood obtained from fingertips did not differ significantly from blood in the cannulated forearm vein. Free fatty acids (FFA) in serum were determined colorimetrically (Duncombe 1964) with palmitic acid as standard. Glycerol (Eggstein & Kreutz 1966) and alanine (Williamson 1974) in serum and lactate (Gutmann & Wahlefeld 1974) in blood were determined by enzymatic spectrophotometric methods and β-hydroxybutyrate and pyruvate by enzymatic fluorometric micromethods (Olsen 1971).

Muscle biopsies obtained after exercise were frozen in liquid nitrogen within 10 sec from the end of an exercise bout and stored at −80°C until analysis. About 10 mg of frozen tissue was divided into five pieces and weighed at −20°C on Perkin-Elmer microbalance to the nearest 0.01 mg. Metabolites were extracted during 15 min in 3 N $HClO_4$ at 4°C. After neutralization with $KHCO_3$ (2 mol l) on dry ice the test tubes were centrifuged in the cold. Glucose 6-phosphate glucose 1-phosphate, glucose lactate and pyruvate were determined on the supernatant by enzymatic fluorimetric micromethods (Lowry & Passonneau 1973). 1 ml HCl (1 mol l⁻¹) was added to the residue and the glycogen was hydrolyzed during 2 h at 100°C. Determination of glucose in the hydrolysates was carried out fluorimetrically (Lowry and Passonneau 1973) and the glycogen concentration is presented as mmol glucosyl units kg⁻¹ (wet weight). For determination of the protein concentration about 7 mg of muscle tissue were heated for 30 min at 100°C in 1 ml 1 N NaOH. The protein concentration was determined spectrophotometrically with the Folin phenol reagent (Lowry et al 1951) with bovine serum albumin as the standard. To estimate the precision of the biopsy technique duplicate analyses were performed on five pieces cut from a 31 mg biopsy of human muscle and on 10 pieces cut from rat leg muscle weighing 80 mg. Mean values (mmol kg wet weight) and coefficients of variation for the different analyses on human and rat muscle were: Glucose 6-phosphate 0.6, 11.5 % 3.29 29 % glucose 1-phosphate undetectable amounts glucose 0.7 32 % 0.7 17 % lactate 2.9 14.5 % 13.3 12.8 % pyruvate 0.363 33 % 0.368 58 % glycogen 122, 7.5 % 36.3 7.3 %. The coefficients of variation of the chemical part of the procedures were below 3 % except for the coefficient of variation of the pyruvate analysis which was 29 %. In

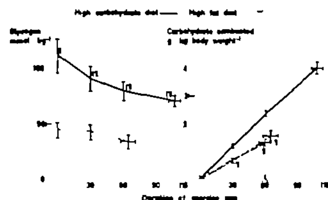


Fig 1 The effect of different diets on the carbohydrate combustion and muscular glycogen concentration during exercise Mean values (± S E) of glycogen in the vastus lateralis muscle (expressed as mmol glucosyl units per kg wet weight) and amount of carbohydrate combusted in seven subjects running on a treadmill In the postabsorptive state each subject exercised three times having eaten through 4 days a diet rich in either carbohydrate or fat Glycogen concentrations were only measured in one of the two experimental series including a fat enriched diet + denotes value significantly different ($P<0.05$) from preexercise (rest) value ↑ denotes significant difference between experiments including different diets

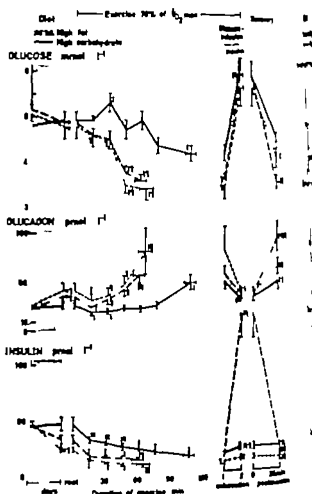


Fig 2 The effects of different diets of exercise and of infusion of glucose and insulin during exercise on glucose pancreatic glucagon and insulin in plasma In the postabsorptive state seven subjects ran three times three weeks apart on a treadmill having eaten in advance a diet rich in either carbohydrate or fat (two experimental series) At exhaustion (exhaustion 1) a 10 min rest was allowed. Then the subjects were encouraged to run again as long as possible (to exhaustion) while glucose or both glucose and insulin (in one of the experimental series with fat diet designated --) concentrations were restored by infusion Values are mean ± S E. denotes significant difference ($P<0.05$) between values obtained before and after a diet period + denotes value significantly different from preexercise (rest) value ‡ denotes value significantly different from value at exhaustion 1 ‖ denotes value significantly different from value at exhaustion 2] denotes significant difference between experiments including different diets

the study all chemical analyses were performed at least in duplicate Enzymes and cofactors were from Boehringer Mannheim

Statistical evaluation of the data was made by means of correlation analysis and by means of Wilcoxon s non parametric ranking test and the *t*-test for paired comparisons (Snedecor & Cochran 1965) Differences were considered to be significant if *P* values of less than 0 05 were obtained with both these tests Concentrations obtained at exhaustion in F_1-and CH-expts (n=14) have been used for correlation analysis when something else is not explicitly stated The cited correlation coefficients are significant on the 5% confidence level

RESULTS

Effects of the different diets

Except for hematocrit [46.?±0 9% mean and S E (F_1-expts) significantly higher than 44 1±0 8 (CH expts) and 43 5±0 9 (F_2-expts)] and FFA [significantly higher in F than in F_2-expts (Fig. 4)] values obtained immediately before each of the three diet periods were similar The energy intake was similar ($P>0.05$) in periods with fat enriched diet [2 492± 159 kcal ?4 h mean and S E (F -expts) and ?790±90 (F_2-expts)] and in periods with carbohydrate enriched diet (2 904±195) However during the diet periods a significant loss of body weight averaging 2 kg took place in fat expts whereas the 0 6 kg weight loss in carbohydrate expts was insignificant After the 4 days of diet the muscular glycogen concentration wa markedly higher in CH than in F-expts (Fig. 1) and a mild

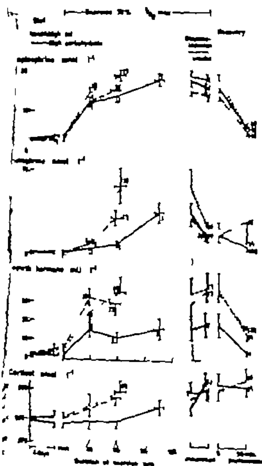


Fig. 3. The effects of different diets, of exercise and of infusion of glucose and insulin during exercise on hormone concentrations in plasma. For further explanation see Fig. 2.

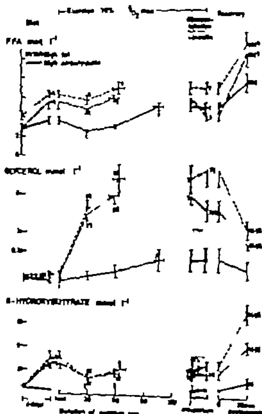


Fig. 4. The effects of different diets of exercise and of infusion of glucose and insulin during exercise on the concentrations of FFA and glycerol in serum and of β-hydroxybutyrate in blood. For further explanation see Fig. 2.

ketonuria had developed in F-expts. The *R* value (respiratory exchange ratio) was at rest 0.74±0.03 after fat diet and 0.84±0.03 after carbohydrate diet (*P*<0.02). Fat diet resulted in decreases in "basal" plasma concentrations of insulin, glucose and alanine, and in increases in the concentrations of glucagon, GH, FFA, glycerol and β-hydroxybutyrate (Figs. 2–4). Carbohydrate diet resulted in small increases in norepinephrine and GH concentrations in plasma in the "basal" state (Fig. 3) and in increases in the concentrations of lactate and alanine too (Fig. 5). At the end of the diet periods hematocrit values (47.8±1.0% (F), 46.2±0.6% (CH) and 45.8±0.6% (F)) had always increased slightly (*P*<0.05). The changes in alanine and insulin during the diet period correlated mutually (*r*=0.73).

Responses to exercise

Metabolic rate (kcal min⁻¹) (as calculated from oxygen uptake and *R* value) and oxygen uptake (69.6±1.7 (F_1-expts.), 69.7±0.9 (CH-expts.) 68.8±1.5 (F_2-expts.)% of individual $\dot{V}_{O_2max}$, *n*=7) were constant during all exercise bouts. However the total work time to exhaustion was much longer in CH-expts. (106±5 min) than in F (64±6) and F_2-expts. (59±6). Heart rate increased from 83±3 (F_1), 78±4 (CH) and 86±2 (F_2) beats min⁻¹ at rest to 171±2 (F_1), 163± (CH) and 169±1 (F_2) in the 30th min of exercise. Heart rate rose gradually throughout exercise and was at exhaustion not significantly different in the 3 expts. (181±3 (F), 178±2 (CH) and 176±2 (F)).

In F-expts. the carbohydrate combustion rate during exercise (calculated from O_2 uptake and R value measurements to be 47±4 (F_1) and 49±3

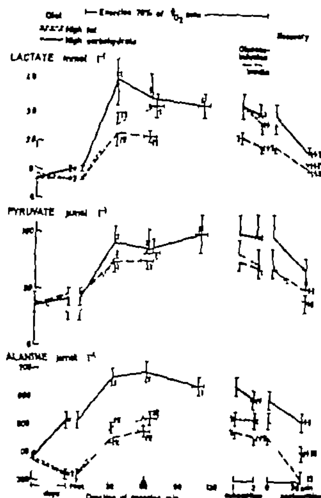


Fig 5 The effects of different diets of exercise and of infusion of glucose and insulin during exercise on the concentrations of lactate and pyruvate in blood and of alanine in serum For further explanation see Fig

(F)% of total energy output per min in the 20th–22nd min) was significantly lower than in CH-expts (83±4%) (Fig 1) Furthermore compared with CH-expts the concentration of glucose in plasma declined more rapidly during exercise in F-expts (Fig. 2) This accelerated decrease of glucose concentrations after intake of a fat diet was accompanied by more rapid increases during exercise in the plasma concentrations of glucagon (Fig 2) epinephrine GH and cortisol (Fig 3) than after a carbohydrate diet During exercise norepinephrine concentrations in plasma always increased progressively above values at rest the responses to exercise after the different diets did not differ for certain (Fig 3) Insulin concentrations in plasma always declined gradually during exercise (Fig) At identical times the concentrations were higher in CH than in F-expts

The concentration of FFA in serum did not change during exercise in F-expts but remained higher than in CH-expts in which an initial decrease was followed by an increase above the concentration at rest (Fig 4) The serum glycerol concentration gradually increased during exercise in all expts but most in F-expts (Fig. 4) The concentration of β-hydroxybutyrate in blood in F-expts was during exercise lower than at rest but still higher than in CH-expts (Fig 4) The blood and serum concentrations of lactate pyruvate and alanine increased during exercise Lactate and alanine concentrations were higher in CH than in F-expts whereas pyruvate concentrations did not differ significantly (Fig 5) Hematocrit values did not change significantly during exercise Comparing the two experimental series with fat diet the responses of hormones and metabolites in plasma to diet and exercise were remarkably similar However hematocrit and alanine concentrations were throughout exercise significantly higher in F_I- than in F_{II}-expts Furthermore at exhaustion carbohydrate combustion rate lactate and FFA were higher and insulin lower in F than in F -expts

In the vastus muscle the concentration of glycogen gradually decreased during exercise in CH-expts but remained higher than the unchanged concentration in F_I-expts (Fig 1) The decrease in glycogen concentration in CH-expts was accompanied by increases in muscular glucose and lactate content (Table 1) The calculated percentage[1] of muscle water in equilibrium with the glucose of plasma water increased in CH-expts from 33±8% at rest to 58±9% (mean and S E) after 30 min of exercise ($P<0.1$) at which time it differed ($P<0.05$) from the value in F -expts (27±7%) In F -expts the apparent glucose distribution space increased only insignificantly during exercise (from 31±8% at rest to 40±5% at exhaustion) The glucose 6-phosphate concentration in muscle tended to decrease

At rest the concentrations f carbohydrate metabolites were lightly higher than mostly reported Apart from possible influence of the diet this discrepancy may be scribed to the fact that the subject in the present study had been tanding, not lying during the 10 min preceding muscle samplin

[1] Calculated as (C_m/C_p) 100 where C and C are the calculated glucose concentrations in muscle and plasma water respectively a suming a water content of 76 and 94% for muscle and pl sma respectively

Table 1 *Muscle metabolites during exercise after different diets*

	Rest	30 min exercise	60 min exercise	Exhaustion
Glucose, μmol kg⁻¹				
CH	1.3±0.3	2.4±0.3	1.9±0.4	1.7±0.2*
F	1.2±0.3	1.0±0.2		1.1±0.1
Glucose-6P μmol kg				
CH	1.4±0.3	1.1±0.2	0.8±0.1	0.6±0.1
F	1.6±0.4	1.1±0.2		0.9±0.1
Lactate μmol kg				
CH	5.1±0.8	8.1±0.9*	7.4±1.4	5.5±0.7
F	3.8±0.9	4.6±0.9		6.7±1.2
Pyruvate μmol kg				
CH	207±48	229±47	239±33	14±42
F	184±54	193±27		251±32
Protein mg g				
CH	183±11	178±6	200±8	184±7
F	207±5	185±6		197±13

Values are mean ± S.E. (n=7). The metabolites were measured in wet muscle tissue[1]
CH carbohydrate diet F = fat diet. Exhaustion was reached after 106±5 min in CH-experiments and after 64±6 min in F-experiments
Value significantly different from pre-exercise value
Significant difference between F and CH-experiment
Value significantly different from pre-exercise value on the 10% probability level ($P<0.1$)

during exercise while pyruvate and protein concentrations did not change significantly (Table 1). At exhaustion the subjects always reported an overall feeling of fatigue being especially pronounced in the calves. In the fat experiments also symptoms of hypoglycemia (weakness irritability mental confusion nausea, hunger cold sweating and disturbed co-ordination) were noticed at exhaustion.

Glucagon correlated with the concentrations in plasma of glucose ($r=-0.66$) insulin ($r=-0.64$) epinephrine ($r=0.57$) and GH ($r=0.56$) and epinephrine cortisol and GH correlated positively ($P<0.05$) with each other. Glucagon epinephrine cortisol and GH correlated negatively ($P<0.05$) with muscular glycogen at exhaustion. Glycerol correlated with epinephrine ($r=0.54$) and with insulin (0.5, $P<0.1$). After 30 min of exercise the decrease in muscular glycogen correlated with both the increase in muscular glucose (=0.63) and the increase in lactate ($r=0.76$) and these changes were correlated mutually (=0.69) and the latter with the decrease in glucose 6-phosphate ($r=$ 0.64). Simultaneously the muscular lactate correlated with blood lactate (=0.58) which in turn correlated with blood pyruvate ($r=0.71$) and alanine (0.60). At exhaustion in F_r- and CH expt ($n=7$) insignificant positive correlations existed between glucose 6-phosphate and plasma glucose as well as muscular glycogen.

Glucose infusion during continued exercise

As judged from reflectance meter readings the glucose concentration in capillary blood was stable on the preexercise level within 4 min of glucose infusion. Although symptoms of hypoglycemia disappeared the subjects were only able to run for further 17.8±0.3 min (F_1-expts.) 15.8±1.6 min (CH-expts.) and 17.5±0.5 min (F_r-expts.). The average rates of glucose infusion necessary to restore and maintain the blood glucose concentration were significantly higher when both glucose and insulin were infused (1.30±0.09 g min⁻¹ F_r-expts.) than when only glucose was infused (0.83±0.08 g min (F_r-expts.) and 0.77±0.06 g min (CH-expts.).

As glucose concentrations were restored plasma glucagon declined to preexercise levels (Fig. 2) and plasma epinephrine, too, was markedly reduced (Fig. 3). During glucose infusion the plasma concentrations of norepinephrine GH and cortisol did not decrease (Fig. 3) and insulin concentrations remained depressed in F_r- and CH-expts. (Fig. 2). As shown in Figs. 4 and 5 the concentrations of fat and carbohydrate metabolites in plasma were not convincingly changed by infusion of glucose alone.

Comparison between F_1 and F_2-expts reveals that acute elevation of plasma insulin above basal levels *did not influence secretion of the other hormones* (Figs 2 and 3) The concentrations of FFA glycerol and β-hydroxybutyrate *were reduced by* insulin (Fig 4) During the infusions hematocrit decreased slightly in F-expts (from 47 5±0 6% to 46 5±0 6% (F_1) and from 46 3±0 7% to 45 1±0 7% (F_2)) whereas heart rate never changed significantly

The 30 min recovery period

In the recovery periods *following glucose or (glucose + insulin)* infusions plasma glucose concentrations again decreased below preexercise levels Plasma glucagon increased above resting levels to mean concentrations which like mean epinephrine and GH concentrations (Fig 3) were inversely related to the mean glucose concentrations (Fig ?) *The plasma insulin concentration* decreased after the end of insulin infusion (F_2-expts) to a value similar to the unchanged low insulin concentrations in F_1 and CH-expts (Fig 2) Norepinephrine concentrations decreased after exercise but remained like epinephrine concentrations above preexercise levels (Fig. 3) Cortisol concentrations remained high after exercise whereas GH concentrations decreased significantly (Fig 3) In the recovery period FFA and β-hydroxybutyrate increased while glycerol concentrations decreased (Fig. 4) the mean values being inversely related to mean insulin concentrations Just as the plasma concentrations of these fat metabolites throughout the entire experimental course were *higher* in F than in CH-expts the concentrations of the carbohydrate metabolites lactate pyruvate and alanine declining in the postexercise period remained higher in CH than in F-expts (Fig. 5) 30 min after exercise heart rate (109±3 beats min in F and 122±4 in CH-expts) and mean oxygen uptake *(148% and 134% of preexercise values* in CH and F-expts respectively) were still significantly increased In CH-expts. R value declined in the postexercise period to values below the preexercise value Hematocrit did not change 30 min after exercise in F and *CH-expts. (n=14)* β-hydroxybutyrate correlated with epinephrine *(r=0 63) and with* insulin ($r=-0$ 80) After termination of the insulin infusion the increases in glucagon and epinephrine concentrations correlated mutually ($r=0$ 67 $P<0$ 1) and both these hormones correlated with plasma glucose ($r=-0$ 67 ($P<0$ 1) and $r=-0$ 79 respectively)

DISCUSSION

Regulation of hormonal secretion during exercise

The importance of the plasma glucose concentration The present study has demonstrated that the hormonal response to prolonged exercise depends upon the preceding diet After intake of a fat enriched diet the glycogen content in muscle (and probably liver Hultman & Nilsson 1971) was markedly lower than after intake of a diet rich in carbohydrate (Fig 1) The resulting more rapid decline in plasma glucose concentration during exercise in expts with fat diet was accompanied by faster increases in the concentrations of glucagon epinephrine GH and cortisol in plasma (Figs 2 and 3)—a finding which suggests that glucose sensitive receptors are able to modulate the hormonal response to prolonged exercise This suggestion is further supported by the effects of glucose infusion When plasma glucose concentrations were restored by exogenous glucose in the final 15 min of exercise plasma epinephrine and glucagon concentrations decreased markedly glucagon concentrations even to preexercise levels irrespective of the preceding diet (Figs 2 and 3) The decreases in the concentrations of these hormones during glucose infusion were due to the glucose infusion and were not a consequence of the 10 min rest period which preceded the infusion This is so since we know from the present and our previous studies of prolonged running divided by short breaks that epinephrine and glucagon concentrations do not decrease during a 10 min intermission and a following 15 min run without infusion (Galbo et al 1975 1976 1977*a* 1977*b*) The plasma concentrations of GH and cortisol hormones which have considerably longer biological half-lives than epinephrine and glucagon did not decrease during the short lasting glucose infusion This does not necessarily mean that the plasma glucose concentration is a minor *determinant of the response of these hormones to exercise* A delay may be inherent in the stimulus secretion coupling and—although not indicated by our data—GH and cortisol concentrations might have increased further if the last run had not been accom-

...led by glucose infusion. After termination of ...cise and infusion glucose concentrations de...eased again while glucagon concentrations in...eased to values which, relati e to glucose con...ntrations, were not lower than those obtained at haustion I (Fig. 2) From this and the above find...gs it is reasonable to conclude that the glucose ...ncentration in plasma is the determinant of the ...cagon response to exercise. 30 min postexercise ...so the concentrations of GH and epinephrine in ... were inversely related to glucose concentra... but the hormone concentrations were lower ...lative to glucose concentrations than at exhaus...on I (Figs. 2 and 3). This indicates that the glucose ...ncentration in plasma is not the sole determinant ...f the GH and epinephrine responses to heavy ...xercise. This conclusion is further substantiated ...y the fact that after 30 min of exercise in expts. ...ith carbohydrate diet the concentration of both ...ese hormones had increased in the face of an ...crease in plasma glucose concentration. The per...sistence of high cortisol concentrations 30 min after ...xercise at a time when glucose concentrations ...gain were below values obtained at rest, but not ...wer than concentrations at exhaustion I is com...patible with the assumption that decreased glucose ...oncentrations significantly enhances cortisol sec...etion during exercise.

Evidence for an influence of plasma glucose con...centrations on the hormonal response to exercise in man has previously been published. Thus the rise in glucagon and epinephrine concentrations was mar...kedly reduced when euglycemia was maintained by glucose infusion throughout exercise (Galbo ...hristensen & Holst 1977*b*) Furthermore the rise ...n glucagon concentration in plasma was abolished ...hen glucose was ingested prior to (Ahlborg & Felig 1977) or during exercise (Luyckx Pirnay & Lefebvre 1978, Ahlborg & Felig 1976) Also the rise of plasma GH during moderate exercise has been ...hown to be preventable by carbohydrate ingestion (Hunter Fonseka & Passmore 1965) and by glucose infusion causing hyperinsulinemia and hyper...glycemia (Hansen 1971)

The importance of insulin On the whole during exercise the concentration of insulin in plasma was inversely related to the concentrations of the other hormones (Figs. 2 and 3). However a sensitizing effect of a decrease I insulin concentrations on the glucose-sensitive cells modulating the hormonal re...sponse to exercise was not proved, since exogenous insulin + glucose did not depress the concentra...tions of the other hormones more than exogenous glucose alone. It could be argued that the sampling of blood during the infusions was too infrequent to demonstrate such an effect. Low insulin concentra...tions might especially act synergistically with low glucose concentrations in plasma. Accordingly a more frequent sampling of blood might have re...vealed a more rapid decrease in glucagon and epinephrine concentrations during restoration of both insulin and glucose concentrations than during restoration of glucose concentrations only One could also postulate that the insulin infusion was too brief or the insulin concentrations achieved dur...ing insulin infusion too low to restore insulin-mediated cellular glucose turnover in the cells regu...lating hormonal secretion. In diabetics normaliza...tion of the exaggerated growth hormone response to exercise requires a preceding strict insulin treat...ment (Hansen 1971*b*) It is possible that the hypoin...sulinemic state induced by fat diet (Fig. 2) in some respect is similar to diabetes and that it was not corrected completely by the short-lasting insulin infusion. Furthermore a decrease in insulin con...centration in peripheral blood probably reflects a much larger decrease in insulin concentration in the fluid bathing the pancreatic glucagon secreting A cells Only about 1% of the blood in the pancreatic vein has passed through the islets of Langerhans and insulin is extracted during hepatic transit. Ac...cordingly the restoration of insulin concentrations in peripheral blood by insulin infusion during exer...cise possibly was not accompanied by an increase of insulin concentrations to resting values at the A cells. An incomplete restoration of insulin effects on the A cells might explain why glucagon concen...trations at the end of the infusions still were 43% above the minimum values obtained 15 min after the beginning of exercise (Fig. 2). Alternatively this finding might be due to the half-life of glucagon being too long in proportion to the infusion periods to allow complete removal of glucagon in excess during these periods.

Th importance of sympathetic activity In the rat the exercise-induced increase in plasma glucagon concentration is mainly caused by increased sym...patho-adrenal activity (Galbo et al 1978). How...ever the present study does not indicate a corre...sponding influence of the sympatho-adrenal system in man. During exercise as judged from norepine...phrine concentrations in plasma, nor-adrenergic

sympathetic nervous activity did not differ with certainty in expts with different diets and did not decrease significantly during glucose infusion (Fig. 3) Thus the enhancement after the fat diet of the glucagon response to exercise and the decline of glucagon concentrations during glucose infusion were probably not secondary to changes in sympathetic nervous stimulation of the pancreatic A cells Glucagon and epinephrine concentrations varied in parallel during exercise in the different expts However the lack of parallelism between the changes in the concentrations of these two hormones in the postexercise period (Figs 2 and 3) indicate that the changes in glucagon concentrations most likely were due to direct effects of glucose on the A cells and not mediated via epinephrine Further support to this view comes from the fact that the glucagon response to hypoglycemia is normal in sympathectomized (Palmer et al 1976) and in adrenalectomized (Ensinck et al 1976) man and from the finding that the glucose–glucagon relationship in exercising man is not changed by adrenergic blocking drugs (Galbo et al 1976 Galbo Christensen & Holst 1977*a*) It is unlikely that glucose infusion changed the *elimination* of glucagon Thus the marked decline of glucagon concentration upon glucose infusion strongly indicates that the exercise induced increase in glucagon concentrations in plasma reflects an increased A cell *secretion*

The present study supports the assumption (Galbo Christensen & Holst 1977*a*) that an inhibiting sympathetic nervous activity rather than plasma glucose concentrations determines plasma insulin concentrations during exercise Early during exercise in spite of unchanged or increased glucose concentrations insulin levels decreased at the same time as norepinephrine concentrations increased Furthermore when glucose was infused norepinephrine concentrations did not decrease and insulin concentrations remained low (Figs 2 and 3)

Muscular metabolism during exercise

Accumulation of glucose and lactate The effect of different diets on the muscular concentrations of carbohydrate metabolites have not previously been studied during prolonged exercise In the present study biopsies were taken from the vastus lateralis muscle No other muscle has been shown to be a significantly better representative of the working muscles during exhaustive running (Costill et al 1971 Karlsson & Saltin 1971) We found that the average distribution space for glucose in muscle reached its highest value (58% of muscle water) after 30 min of exercise in expts with carbohydrate enriched diet (Table 1) At least at that time the distribution space was higher than the extracellular space (determined at rest as the raffinose space to be 20–30% in the rat depending on the type of muscle (Kipnis Helmreich & Cori 1959) determined as the chloride space in man to be 14 and 17% in resting and exercised muscle respectively (Ahlborg et al 1967) the negligible change from rest to exercise being in accordance with the constancy of muscular protein concentrations found in the present study (Table 1)) The concentration of intracellular free glucose is generally considered to be very low at rest (Elbrink & Bihler 1975 Rennie & Holloszy 1977) but it has previously been demonstrated to increase when perfused rat skeletal muscle is stimulated to contract (Rennie & Holloszy 1977) The finding of an accumulation of intracellular glucose during exercise at a time when carbohydrate oxidation was increased may be explained by a relative enhancement of glucose transport across the cell membrane (Elbrink & Bihler 1975 Rennie & Holloszy 1977) but may also be due to intracellular production of glucose from glycogen (Saha Lopez Mondragon & Narahara 1968 Wahren 1970) and also to a decreased glucose phosphorylation due to an inhibition of hexokinase by glucose 6-phosphate derived from glycogen The concentration of glucose 6-phosphate did in fact not increase during exercise (Table 1) But compared with expts with fat enriched diet the intracellular glucose concentration was higher during exercise in expts with carbohydrate enriched diet in which also the estimated mean rate of muscular glucose uptake as well as the mean rate of muscular

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Concentration of glucose in intracellular water (mmol l) = [concentration in wet muscle tissue (mmol kg) − concentration in plasma water (mmol l) × amount of extracellular water per kg wet muscle tissue (l kg)]/(amount of intracellular water per kg wet muscle tissue (l kg))

Mean rate of muscular glucose uptake (U) = (total amount of carbohydrate combusted − glycogen depletion per kg active muscle × estimated active muscle mass − increase in glucose concentration in intracellular water × amount of intracellular water in active muscle mass + 0.5 × (sum of increases in concentrations of lactate pyruvate and alanine in plasma water × amount of extracellular

glycogen breakdown (Fig. 1) were higher. Accompanying the higher rate of carbohydrate oxydation (Fig. 1) the exercise-induced increase in lactate concentrations in muscle (Table 1) and blood (Fig. 5) was higher after carbohydrate diet than after fat diet. Lactate production during exercise has in the past been considered simply a consequence of hypoxia in the working muscles (Elbrink & Bibler 1975). However in the exercising muscles the availability of oxygen (and the exit of lactate over the cell membranes) hardly was lower after carbohydrate enriched diet than after fat enriched diet. Accordingly the present experiments support the view that the magnitude of lactate production rather depends upon the balance between the rate of glycolysis and the capacity for alternative elimination of pyruvate and NADH (Holloszy & Booth 1976).

Effect of exogenous glucose on performance. Low glucose concentrations in plasma (Bøje 1935, Christensen & Hansen 1939, Bergstrom et al. 1967) and low muscular glycogen concentrations (Ahlborg et al. 1967, Bergstrom et al. 1967, Karlsson & Saltin 1971) have previously been found at exhaustion after prolonged exercise. Furthermore elevation of the initial muscular and hepatic glycogen concentrations by intake of a diet rich in carbohydrate (Bøje 1935, Christensen & Hansen 1939, Bergstrom et al. 1967, Karlsson & Saltin 1971) as well as ingestion of glucose at exhaustion i[n] expts. with fat enriched diet (Bøje 1935, Christensen & Hansen 1939) have been shown to increase performance time. These findings indicate that continuation of exercise at a given intensity requires that the carbohydrate combustion exceeds a certain lower limit. A lower limit, which probably depends upon the supply of FFA to the working muscles since the carbohydrate combustion as well as plasma glucose and muscular glycogen concentrations at exhaustion are higher in expts. with carbohydrate enriched diet than in expts. with fat enriched diet (Figs. 1 and 2, Bøje 1935, Bergstrom et al. 1967), the plasma concentration of FFA being higher in the latter (Fig. 4). At exhaustion in the present study low muscular glycogen concentration, decreased glucose gradient between plasma and muscle water and decreased muscular glucose 6-phosphate concentration (Table 1) indicated that the glucosyl unit turnover in the vastus muscle was lower than earlier during exercise. The simultaneous increase in lipolysis indicated by increased FFA and glycerol concentrations possibly did not completely compensate for the decrease in glucosyl flux and in consequence an energy crisis developed in the working muscles.

In contrast to the findings in the above mentioned earlier studies (Bøje 1935, Christensen & Hansen 1939) infusion of glucose did not eliminate the fatigue in the present study. This discrepancy may be due to differences between the different studies as regards the cause of exhaustion. In fact in the earlier studies the improvement of performance by exogenous glucose was ascribed entirely to disappearance of cerebral hypoglycemia, because the overall carbohydrate combustion rate, as judged by R-value measurements, did not increase after glucose administration (Bøje 1935, Christensen & Hansen 1939). In our expts. with fat enriched diet, however, cerebral hypoglycemia was not the major cause of exhaustion, since glucose infusion eliminated the hypoglycemic symptoms but not the fatigue. Although a diet rich in fat was consumed before exercise in the earlier as well as in the present study a muscular lack of energy possibly limited performance only in the present study. This might be the case since in the present study but not in the earlier studies intake of a fat enriched diet was preceded by heavy exercise, a regimen known to deplete muscular glycogen stores more than mere intake of fat enriched diet (Hultman 1967). Alternatively, exhaustion was in the earlier as well as in the present expts. caused by a muscular lack of energy and the fact that glucose administration improved performance only in the earlier expts. was due to a higher supply of exogenous glucose relative to the minimal glucose need in the earlier than in the present expts. (200 g glucose ingested in 10 min and an estimated previous glucosyl unit turnover of 1.2 g min in the earlier studies vs an exogenous supply of glucose corresponding to 44 and 26% respectively of previous glucose-unit turnover in the present F_T- and CH-expts.) The conclusion that deficiency of energy substrates in working muscles rather than neuroglucopenia is decisive for exhaustion has previously been drawn from studies, in

ater (0.2 body weight) ... ions of increases in concentrations of lactate and pyruvate in intracellular water ... amount of intracellular water in active muscle mass))/total work time. Mean rate of hepatic glucose production = ... decrease in concentration of glucose in plasma water ... amount of extracellular water ... work time

sympathetic nervous activity did not differ with certainty in expts with different diets and did not decrease significantly during glucose infusion (Fig. 3) Thus the enhancement after the fat diet of the glucagon response to exercise and the decline of glucagon concentrations during glucose infusion were probably not secondary to changes in sympathetic nervous stimulation of the pancreatic A cells Glucagon and epinephrine concentrations varied in parallel during exercise in the different expts However the lack of parallelism between the changes in the concentrations of these two hormones in the postexercise period (Figs 2 and 3) indicate that the changes in glucagon concentrations most likely were due to direct effects of glucose on the A cells and not mediated via epinephrine Further support to this view comes from the fact that the glucagon response to hypoglycemia is normal in sympathectomized (Palmer et al 1976) and in adrenalectomized (Ensinck et al 1976) man and from the finding that the glucose–glucagon relationship in exercising man is not changed by adrenergic blocking drugs (Galbo et al 1976 Galbo Christensen & Holst 1977*a*) It is unlikely that glucose infusion changed the elimination of glucagon Thus the marked decline of glucagon concentration upon glucose infusion strongly indicates that the exercise induced increase in glucagon concentrations in plasma reflects an increased A cell secretion

The present study supports the assumption (Galbo Christensen & Holst 1977*a*) that an inhibiting sympathetic nervous activity rather than plasma glucose concentrations determines plasma insulin concentrations during exercise Early during exercise in spite of unchanged or increased glucose concentrations insulin levels decreased at the same time as norepinephrine concentrations increased Furthermore when glucose was infused norepinephrine concentrations did not decrease and insulin concentrations remained low (Figs 2 and 3)

Muscular metabolism during exercise

Accumulation of glucose and lactate The effect of different diets on the muscular concentrations of carbohydrate metabolites have not previously been studied during prolonged exercise In the present study biopsies were taken from the vastus lateralis muscle No other muscle has been shown to be a significantly better representative of the working muscles during exhaustive running (Costill et al 1971 Karlsson & Saltin 1971) We found that the average distribution space for glucose in muscle reached its highest value (58% of muscle water) after 30 min of exercise in expts with carbohydrate enriched diet (Table 1) At least at that time the distribution space was higher than the extracellular space (determined at rest as the raffinose space to be 20–30% in the rat depending on the type of muscle (Kipnis Helmreich & Cori 1959) determined as the chloride space in man to be 14 and 17% in resting and exercised muscle respectively (Ahlborg et al 1967) the negligible change from rest to exercise being in accordance with the constancy of muscular protein concentrations found in the present study (Table 1)) The concentration of intracellular free glucose is generally considered to be very low at rest (Elbrink & Bihler 1975 Rennie & Holloszy 1977) but it has previously been demonstrated to increase when perfused rat skeletal muscle is stimulated to contract (Rennie & Holloszy 1977) The finding of an accumulation of intracellular glucose during exercise at a time when carbohydrate oxidation was increased may be explained by a relative enhancement of glucose transport across the cell membrane (Elbrink & Bihler 1975 Rennie & Holloszy 1977) but may also be due to intracellular production of glucose from glycogen (Sahu Lopez-Mondragon & Narahara 1968 Wahren 1970) and also to a decreased glucose phosphorylation due to an inhibition of hexokinase by glucose 6-phosphate derived from glycogen The concentration of glucose 6-phosphate did in fact not increase during exercise (Table 1) But compared to expts with fat enriched diet the intracellular glucose concentration[1] was higher during exercise in expts with carbohydrate enriched diet in which also the estimated mean rate of muscular glucose uptake as well as the mean rate of muscular

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[1] Concentration of glucose in intracellular water (mmol l⁻¹) = [concentration in wet muscle tissue (mmol kg⁻¹) − concentration in plasma water (mmol l⁻¹) × amount of extracellular water per kg wet muscle tissue (l kg⁻¹)]/(amount of intracellular water per kg wet muscle tissue (l kg⁻¹))

Mean rate of muscular glucose uptake (U) = (total amount of carbohydrate combusted − glycogen depletion per kg active muscle × estimated active muscle mass − increase in glucose concentration in intracellular water × amount of intracellular water in active muscle mass + 0.5 × (sum of increases in concentrations of lactate pyruvate and alanine in plasma water × amount of extracellular

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which an intracarotid glucose infusion given at exhaustion did not restore the work capacity in dogs (Nazar Brzezińska & Kowalski 1972) It may appear strange that performance time was the same when glucose in the present study was infused together with insulin and at a rate corresponding to as much as 74% of the previous carbohydrate combustion rate as it was when a smaller amount of glucose was infused without insulin The explanation may be that "insulin inhibited lipolysis (Fig. 4) and accordingly increased the glucose need But how do we explain that the elimination of muscular fatigue by exogenous glucose during exercise may take place without an increase in carbohydrate combustion rate (Boje 1935 Christensen & Hansen 1939)? Let us assume that primarily resting fibers are recruited when primarily recruited fibers are exhausted due to an insufficient glucosyl turnover Let us in addition assume that an individual feels exhausted when a fixed number of muscle fibers are fatigued or a fixed intensity of innervation is exceeded In agreement with these assumptions glucose administered at exhaustion may be metabolized in and restore the function of the primarily recruited fibers in that way saving activity and glycogen combustion in secondarily recruited fibers and accordingly eliminating fatigue without an increase in carbohydrate combustion rate

CONCLUSION

The present study has shown that in man the rate of carbohydrate combustion is higher during exercise after intake of a diet rich in carbohydrate than after intake of a fat enriched diet probably as a consequence of both higher hepatic glucose output and muscular glycogen breakdown However the exercise induced changes in plasma concentrations of norepinephrine and insulin are similar after the two diets and after the carbohydrate enriched diet the changes in epinephrine glucagon cortisol and growth hormone are delayed in parallel with a slower decline in plasma glucose concentration Hypothetically the regulation of metabolism during prolonged exercise proceedes as follows Impulses from motor centers in the cerebral cortex or from the working muscles elicit a work load dependent activity in the sympathetic nervous system (Galbo Holst & Christensen 1975) which in turn depresses insulin secretion (Galbo Christensen & Holst 1977*a*) These changes in norepinephrine and insulin levels together with the size of the extramuscular fuel depots as well as the state of receptors and enzymatic machinery in these depots determine the primary mobilization of glucose and free fatty acids from liver and fat tissue The mobilized substrates are taken up by the exercising muscles the uptake involving mass effects (Schultz et al 1977 Hagenfeldt & Wahren 1971) and are burned in competition with intracellular energy depots (Galbo Christensen & Holst 1977*b* Hickson et al 1977 Rennie & Holloszy 1977) and in mutual competition (Rennie & Holloszy 1977) Excessively mobilized free fatty acids (Issekutz, Issekutz & Nash 1970) may be stored as muscular triglyceride (Zierler 1976) Initiated by decreasing plasma glucose concentration a sensitive secondary safety back up mechanism operates by increasing the secretion of the fuel mobilizing hormones epinephrine glucagon cortisol and GH

The investigation was supported by grants from The Danish Medical Research Council The P Carl Petersen Foundation Forskningsfonden for Storkøbenhavn, Færøerne og Grønland and The J Weimann Foundation Lisbeth Kall Vibeke Ulrik Kristensen Birthe Knudsen Rikke Grønboldt and E A Richter performed skilled technical assistance Equipment for gas analysis was kindly lent by the Institute of The Theory of Gymnastics, Copenhagen Human monocomponent insulin was kindly provided by NOVO Research Institute Doctor in chief E S Olesen The Department of Nutritional Physiology Kommunehospitalet Copenhagen composed the diets.

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of muscle temperature on maximal muscle strength power output in human skeletal muscles

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BERGH U & EKBLOM B Influence of muscle temperature on maximal muscle strength and power output in human muscles. Acta Physiol Scand 1979 107 33–37 Received 15 Dec. 1978 ISSN 0001-6772. Department of Physiology III Karolinska Institute Stockholm Sweden.

The influence of muscle temperature (T) on maximal muscle strength, power output, jumping, and sprinting performance was evaluated in four male subjects. In one of the subjects the electromyogram (EMG) was recorded from M vastus lateralis M biceps femoris, and M. semitendinosus T ranged from 30.0°C to 39°C. Maximal dynamic strength, power output, jumping and sprinting performance were positively related to T The changes were in the same order of magnitude for all these parameters (4–6 % °C^{-1}) Maximal isometric strength decreased by 2 % × °C^{-} with decreasing T The force–velocity relationship was shifted to the left at subnormal T Thus in short term exercises, such as jumping and sprinting, performance i reduced t low T and enhanced at T above normal primarily as a result of a variation in maximal dynamic strength

Key words Muscle temperature muscle strength, force-velocity physical performance

An elevated muscle temperature is known to improve performance in exercises such as short term bicycling (Asmussen & Bøje 1945) and 100–800 m running (Hogberg & Ljunggren 1947) whereas a subnormal tissue temperature is accompanied by a decrease in performance (Davies et al. 1975). However in these studies no data were given to illustrate the quantitative aspects of these effects Therefore, part of the purpose of this investigation was t study the effect of different levels of especially lowered but also enhanced tissue temperatures on short time physical performance

Furthermore reported data on the effects of different temperatures on muscle strength are conflicting. Thus Asmussen et al (1976) found a significant correlation between muscle temperature and maximal isometric strength whereas Binkhorst et al (1977) concluded that maximal *isometric* strength was independent of muscle temperature Fall (1971), in his review article, stated that maximal isometric strength seemed to be unaffected by temperature On the other hand endurance in submaximal isometric exercise was reported by Edwards et al (197?) to be enhanced at subnormal muscle temperature Binkhorst et al (1977) also reported maximal dynamic strength to be higher at elevated muscle temperature while no data are available about the effects of lowered temperature on maximal dynamic strength. Therefore the aim of this study was to study the quantitative effects of different muscle and core temperatures on static and dynamic maximal strength with special emphasize on the force–velocity relationship and its consequence on sprinting and vertical jump performance

METHODS AND PROCEDURE

Four male subjects aged 24–39 years participated in the study They were physically well-trained and familiar with the laboratory proceedings Muscle fibre composition expressed as the relative number of type I fibres ranged from 45 % to 65 % Temperature may affect the function of the different fibre types differently (Ranatunga, 1977). Therefore it was considered necessary to use subjects with rather similar fibre type distribution. The experimental protocol is illustrated in Fig 1 This protocol was repeated at the following levels of core (T_{oe}) and vastus lateralis muscle temperature (T)

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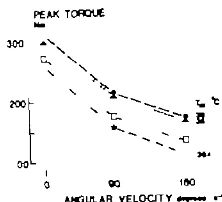
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ig 3 Peak torque as function of angular velocity of the nee joint at different levels of T Average values, =4

ied out at pH 9 4 following alkaline preincubation at pH 03

In one of the subjects an electromyogram (EMG) from of vastus lateralis M semitendinosus, and M biceps emoris was recorded using skin surface electrodes The EMG was evaluated only in regard to the timing of onset and termination of activity of these muscles, relative to one another

RESULTS

e number of subjects was small. Therefore statistical analyses would be of limited value I nstead individual data for maximal dynamic strength 90° × s) sprinting, and jumping performance are shown in F g. Average values are presented in Fig 3 and 4 and in Table 1

Maximal muscle strength Peak torque was positively related to T The changes were 6.5 N×m×°C⁻ (2.1% °C), 9.8 N×m×°C⁻ 4 7% °C), and 8 3 N m×°C⁻ (4.9%×°C) t the angular velocities of 0° 90° and 180° s re spectively Thus, the effect of temperature was greater in dynamic than in isometric exercise

Vertical jump The height of the jump decreased with decreasing T at a rate of 2.1 cm ×°C⁻ (4 2% °C⁻)

Sprinting performance The shortest time in which 10 revolutions could be performed was in ely related to T The work time increased from 10.7 s at T =38.3 to 15.27 at T =31 4°C The increment in the average speed of the periphery of the flywheel was 0.5 m s (4 7% °C) Peak velocity was also positively related to T and changed by 0.51 m× ×°C⁻ (4 4% °C)

Power output During knee extensions the peak power output was positively related to T The rates of the changes were 28.5 W×°C⁻ (5 6%×°C⁻¹) and 15 8 W×°C⁻ (4 9%×°C⁻) at the angular velocities 90° and 180°×s respectively For the first two revolutions of bicycling average power output increased with increasing temperature at a rate of 45 7 W×°C⁻ (5 1%×°C⁻)

EMG The EMG-activity pattern (defined as in Methods") of the M vastus lateralis, M semitendinosus and M biceps femoris were similar regardless of muscle temperature

DISCUSSION

In this study the effect of temperature on jumping and sprinting performance was of the same order of magnitude as indicated by the data reported by Asmussen et al. (1945 1976) According to the latter studies the changes were 4 4%×°C⁻ for jumping (T range 27–40°C) and 5 3%×°C⁻ for sprinting (T range 36–40°C) performance compared to 4.2%×°C⁻ and 5 1%×°C⁻ respectively in the present study

Sprinting and jumping performances were affected in direct proportion to the change in peak torque This implies that temperature changes had little if any effect on coordination in these rather uncomplicated movements. This is also supported by the fact that the EMG-activity patterns were unaffected by temperature

Falls (1972) in his review article stated that maximal isometric strength is unaffected by temperature Thus is in agreement with the data reported by Binkhorst et al (1977). Asmussen et al (1976)

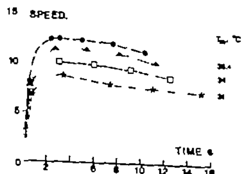


Fig 4 Speed of the periphery of the fly-wheel as function of time at 4 different levels of T Average values =4

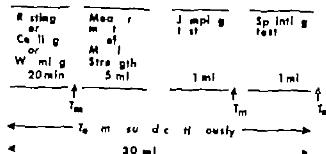


Fig 1 The experimental protocol T_{es}=esophageal temperature T =muscle temperature

	Experimental situations			
	1	2	3	4
T_{es} °C	35–36	35–36	36–37	37–38
T_m °C	30–32	33–35	36–37	38–39

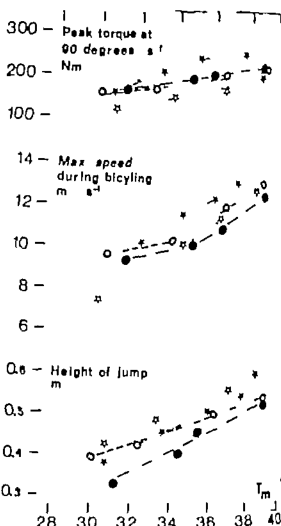


Fig 2 Individual data for peak torque during knee extensions maximal speed obtained during bicycling and the height of the vertical jump at different vastus lateralis temperatures (T).

Elevated body temperatures were achieved by intermittent bicycle ergometer exercise Low muscle temperatures (expts *1 and 2*) were produced by immersing the legs (to trochanter major) in cold water

The order of the experimental situations was varied in order to diminish the influence of a possible training effect with testing day at least 2 days apart

Maximal muscle strength of the left knee extensors was recorded as torque during maximal voluntary knee extension with fixed angular velocities The subjects were sitting in an experimental chair and the lower leg moved the lever arm of an isokinetic dynamometer (Cybex II Lumex *Inc New York)*

Maximal strength was measured at 3 different angular velocities 0° (isometric strength) 90° 180°× s

Jumping performance was determined by a vertical jump The subjects were allowed to start the jump with a *countermovement (knee-flexion) and a downward-upward* arm swing The height of the jump was measured by a measuring tape with one end fastened to the subject waist The attached tape was sliding through a slit with friction just enough to stop the tape at the peak of the jump

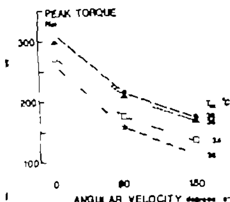
Sprinting performance was evaluated on using a mechanically braked bicycle ergometer All subject performed *20 full pedal revolution (~8.5 kJ) as fast as possible* Time for each revolution wa electrically measured with an accuracy of 0 005 s Time for 2 5 10 and 20 re olutions respectively was recorded *Speed of the* periphery of the fly wheel wa lso calculated at all of the above particular revolutions

Peak power output during knee extensions was calculated according to $P = M \times \omega$ where P=power (W) M=peak torque (N m) and ω=angular velocity (rad × s ¹)

Average power output during the first two revolutions of bicycling was determined according to $P=(w + \quad) \times$ t where P=power w =force nece sary to overcome the frictions between the fly wheel and the friction belt times the distance covered by a point on the periphery of the fly-wheel and =work transferred to the fly wheel when its mass is accelerated [($ =\frac{1}{2} J \times \omega^2$ where J moment of inertia (0 435 × m²) and ω=speed of the fly wheel (rad × s))]

Body temperatures were measured by thermocouples T_{es} in the esophagus at the le el of the heart Muscle temperatures were recorded in M vastus lateralis of the left leg at various depths of 30 t 50 mm and peak the was used (*Saltin et l 1968*) T_m was monitored continuously during the experiment T w measured immediately before the trength mea urement after the j mping te t and after the sprinting test (*cf Fig 1*) T values given in the Results in rel tion to the d fferent tests are means of alues obtained bef re and after the test

Muscle samples from M vastus lateralis were obtained using percutaneou needle biopsy technique (Bergström 196_) The myofibrillar ATP-ase method (Padykula & Herman 1955) s modified by Guth & Samaha (1969) used for muscle fibre clas ification The reaction was ca



...g. 3. Peak torque as a function of angular velocity of the knee joint at different levels of T_m. Average values, n=4.

...ed out at pH 9.4 following alkaline preincubation at pH 6.3.

In one of the subjects an electromyogram (EMG) from M. vastus lateralis, M. semitendinosus, and M. biceps femoris was recorded using skin surface electrodes. The EMG was evaluated only in regard to the timing of onset and termination of activity of these muscles, relative to one another.

RESULTS

The number of subjects was small. Therefore statistical analyses would be of limited value. Instead, individual data for maximal dynamic strength (90° ...) sprinting, and jumping performance are shown in Fig. ... Average values are presented in Fig. 3 and 4 and in Table 1.

Maximal muscle strength. Peak torque was positively related to T_m. The changes were 6.5 N×m×°C (2.1%×°C), 9.8 N×m×°C (4.7%×°C), and 8.3 N×m×°C (4.9%×°C) at the angular velocities of 0°, 90° and 180°×s respectively. Thus the effect of temperature was greater in dynamic than in isometric exercise.

Vertical jump. The height of the jump decreased with decreasing T_m at a rate of ...1 cm×°C (4 ... °C).

Sprinting performance. The shortest time in which 20 revolutions could be performed was inversely releated to T_m. The work time increased from 10.77 s at T_m =38.3 to 15.27 at T_m =31.4°C. The increment in the average speed of the periphery of the flywheel was 0.5 m×s (4.7% °C). Peak velocity was also positively related to T_m and changed by 0.51 m s °C (4.4% °C).

Power output. During knee extensions the peak power output was positively related to T_m. The rates of the changes were 28.5 W×°C (5.6%×°C^{-1}) and 15.8 W×°C (4.9%×°C^{-1}) at the angular velocities 90° and 180°×s respectively. For the first two revolutions of bicycling average power output increased with increasing temperature at a rate of 45.7 W×°C (5.1%×°C).

EMG. The EMG-activity pattern (defined as in "Methods") of the M. vastus lateralis, M. semitendinosus and M. biceps femoris were similar regardless of muscle temperature.

DISCUSSION

In this study the effect of temperature on jumping and sprinting performance was of the same order of magnitude as indicated by the data reported by Asmussen et al. (1945, 1976). According to the latter studies the changes were 4.4%×°C for jumping (T_m range 27–40°C), and 5.3%×°C for sprinting (T_m range 36–40°C) performance compared to 4.2%×°C and 5.1%×°C respectively in the present study.

Sprinting and jumping performances were affected in direct proportion to the change in peak torque. This implies that temperature changes had little if any effect on coordination in these rather uncomplicated movements. This is also supported by the fact that the EMG-activity patterns were unaffected by temperature.

Falls (1972) in his review article stated that maximal isometric strength is unaffected by temperature. This is in agreement with the data reported by Binkhorst et al. (1977). Asmussen et al. (1976)

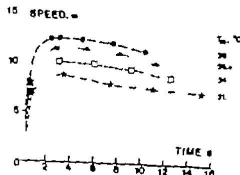


Fig. 4. Speed of the periphery of the fly-wheel as function of time at 4 different levels of T_m. Average values, n=4.

Table 1 *Average values and range for the data obtained at the four different experimental situations*

	Experimental situation			
	1		3	4
Knee extensions				
T_{re} °C	35 8	35 6	37 0	37 9
	35.3–36 4	35.2–36.3	36 8–37.5	36 6–38 4
T_m °C	30 4	33 6	36 1	38 5
	30 0–31.2	3[illegible].8–33.8	35.[illegible]–36.5	37 7–39 0
0°×s peak torque (N×m)	[illegible]	282	301	31[illegible]
	190–302	[illegible]60–310	[illegible]–3[illegible]	289–360
90°×s peak torque (N×m)	148	177	211	[illegible]16
	115–163	158–[illegible]05	181 [illegible]	197 237
90°×s peak power W	233	[illegible]77	331	333
	181–256	[illegible]48–3[illegible]1	[illegible]84–411	309–3[illegible]
180°×s peak torque (N×m)	111	136	169	174
	84–137	126–163	139–201	167 [illegible]
180°×s peak power W	348	428	510	540
	264–430	366–512	436–631	[illegible]–591
Vertical jump				
T_{re} °C	35.8	35 6	36.9	37.8
	35.3–36 4	35 2–36 4	36 7–37 1	37.6–38 3
T_m °C	30 6	33 9	36.2	38 4
	30.0–31	32.3–34 4	35 4–36 9	37 6–39 0
Height m	0.375	0 435	0 493	0 540
	0.325–0 410	0 395–0 450	0 450–0.500	0 510–0 590
Bicycling				
T_{re} °C	35 7	35 6	37.0	37.8
	35.2–36	35.[illegible]–36.3	36.6–37 3	37 6–38.3
T_m °C	31 4	34.3	36.4	38.3
	30 [illegible]–32.3	33.8–34 9	36.1–36.6	37.3–38 [illegible]
Time for [illegible]0 rev s	15[illegible]7	1[illegible] 63	11 3	10.37
	13 19–20 14	11 61–13 65	10 51–1[illegible]3	9 95–10 72
Max speed m×s	8 96	10.33	11 54	1[illegible] 4
	7 40–10 15	10 00–11 45	10 70–12 15	12 [illegible]5–1[illegible] 95
Power W first rev	683	797	921	999
	594–789	75[illegible]–845	834–934	956–1113

found only a minor effect of temperature on maximal isometric strength and in the present study the changes in muscle temperature had only a small and probably insignificant influence in this respect Thus it may be concluded that maximal isometric strength is affected little by changes in muscle temperature

In contrast maximal dynamic strength was markedly dependent on T_m This difference in temperature response may arise from the fact that peak torque can be attained only in a limited part of the range of movement (Thorstensson et al 1976) Thus the time available to attain peak torque is shorter in dynamic than in isometric exercise in the present study the values were only 0 1–0 2 s during dynamic but 2–4 s in isometric contractions At low temperature it might be more difficult to activate the motor units during a short time interval possibly as a result of a lower nerve impulse frequency (Vangaard 1975) Furthermore the speed of chemical reactions is decreased at low temperatures Thus the breaking and formation of the cross-bridges may be considerably delayed at low temperatures resulting in a slower rate of tension

development, even though the maximal tension is little affected by temperature. However, this seems to be contradicted by the fact that the temperature effect was 4–6 %×°C⁻¹ independent of contraction velocity over a wide range of movement (90–30° s⁻¹). The reason for this remarkable independence of speed of contraction is so far obscure.

From the present results it is evident that T_m has a considerable effect on performance in short term dynamic exercise. In contrast, changes in T_m have little if any importance during maximal isometric contractions, while dynamic muscle strength will be influenced considerably by T_m. Finally, the effect of temperature on jumping and sprinting performance is primarily a result of a variation in maximal dynamic strength.

This study was supported by the Research Council of the Swedish Sports Federation (grant 29/77).

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Measured during bicycling.

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;keletal muscle metabolism morphology and function n sedentary smokers and nonsmokers

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ÖRLANDER, J KIESSLING K-H & LARSSON L. Skeletal muscle metabolism, morphology and function in sedentary smokers and nonsmokers. Acta Physiol Scand 1979 107 39–46. Received 29 Dec 1978. ISSN 0001-6772. Institute of Zoophysiology Univer sity of Uppsala, the Department of Animal Nutrition Swedish University of Agricultural Sciences, Uppsala, and the Laboratory for Human Performance National Defence Re search Institut Stockholm, Sweden.

Smokers and nonsmokers of homogeneous population of sedentary men have been compared with respect to skeletal muscle (vastus lateralis) morphological metabolic and functional characteristics. The percentage type I fibres was lower and that of type II B fibres higher in the smokers. Fibre areas were almost equal in the two groups Muscle oxidative capacity was lowered in the smokers as judged from decreased mitochondrial enzyme activities and lowered fibrillar space mitochondrial volume fraction. Isometric and dynamic strengths were lower in the smokers except at the highest velocity of movement tudied Dynamic strengths expressed i relation to isometric strength were similar at all velocities except the highest, where the smokers were relatively stronger Muscular endurance, measured in short-term isometric and dynamic tests, was not differ ent It is suggested that the lowered muscle oxidative capacity and trength in the smokers may be partly consequence of the different fibre type distribution A possibly lower physical activity level and tobacco smoke constituents (e g carbon monoxide) may also be instrumental It is not clear whether the different fibre type distribution in the smokers is an effect of smoking per se or if background factors are responsible.

Key ords Muscle metabolism muscle morphology smoking, strength, tobacco

Tobacco smoking is known to markedly impair the capacity for prolonged exercise as revealed by per formance tests and/or measurements of maximal oxygen uptake (Cooper et al. 1968, Peterson & Kelley 1969 McDonough et al. 1970 Hrubes & Battig 1970 Shaver 1973 Raven et al 1974 Ingemann-Hansen & Halkjaer-Kristensen 1977). The impaired working capacity has been attributed to increased oxygen debt and lactate production in smokers (Chevalier et al. 1963 Krumholz et al. 1964 Krumholz & Hedrick 1972, Reece & Ball 1972) although there are conflicting data (Raven et al. 1974) Carbon monoxide has been implicated as a major etiological agent (Chevalier et al 1966).

The physical working capacity and the metabolic capacity of the working muscles are closely interrelated (Holloszy & Booth 1976) It would therefore seem conceivable that part of the impairment of the working capacity in smokers might be related to effects on muscle There is but scanty information in the literature concerning possible effects of tobacco smoking on skeletal muscle characteristics Some early studies (reviewed by Fischer et al. 1960) on the effect of smoking on muscle strength or efficiency showed variable results (increases de creases or no effect at all). Apparently no studies on muscle morphology or metabolism in relation to smoking have appeared so far

In the present study smokers and nonsmokers of a homogenous population of sedentary men were compared with respect to skeletal muscle mor phological, metabolic and functional characteristics.

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Table I *Anthropometrical characteristics*

Values are means ± S E FFS fat free soft tissue weight n s not significant

Group	No of subjects	Age (y)	Height (cm)	Weight (kg)	FFS (kg)	Skeletal weight (kg)	Body fat weight (kg)	Thigh circumference (cm)
Smokers	18	44±3	179±	78 4±2 6	53 9±1 7	1_4±0.5	10 0±0.8	54 7±0 9
Nonsmokers	25	43±3	180±1	76.8±1 6	53 7±1 3	1 8±0_	9_±0.5	54_ 0 7
P		n s	n s	n s	n s	n s	n s	n s

MATERIAL AND METHODS

Subjects 43 apparently healthy men (18 smokers and 25 nonsmokers) volunteered to participate in the study They were all white-collar workers (employees of an insurance company) and engaged in little or no physical activity during their spare time The basis for acceptance of a subject was the desire to get a subject population as homogeneous as possible with regard to physical activity level Smoking habits were not considered in this selection An oral consent was obtained from each subject after he had been informed of the procedure and possible risks of the experiments.

Informations regarding smoking habits were obtained by using a questionnaire Each subject was asked to specify his weekly tobacco consumption 11 were cigarette smokers and smoked 91 (10–200) cigarettes with or without filters per week. 4 subjects smoked the pipe and consumed 62 (25–125) g of tobacco per week 3 had "mixed" habits smoking both the pipe and cigars or cigarettes It was decided to consider all smokers together as one group

For each subject age height weight skeletal width and skinfold thickness were recorded Body fat weight skeletal weight and fat free soft tissue weight (FFS) were calculated according to von Döbeln (1964) The circumference of the left thigh was measured in a horizontal plane just under the gluteal furrow As shown in Table I the two groups were very similar with regard to all studied anthropometrical variables.

All experiments were performed at 8–11 a.m. and followed a fixed protocol with biopsies preceding strength measurements No restrictions regarding smoking were given but no subject smoked during at least 30 min preceding the experiments

Muscle biopsies Muscle tissue was obtained from the left vastus lateralis by the percutaneous needle technique (Bergström 1962) The specimens were used for histochemistry enzyme assays and electron microscopy

Histochemistry The muscle samples for fibre typing and area determination were trimmed mounted frozen in isopentane cooled by liquid nitrogen and stored at −80°C until analysis

Serial transverse sections (10 μm) were cut with a cryotome at −20°C and the myofibrillar ATPase staining method (Gomori 1941 Padykula & Herman 1955) was used for muscle fibre classification Photographs of the stained sections were taken and classification into type I (slow twitch) and type II (fast twitch) (Engel 1962) was made in all 43 subjects In 34 of the subjects the type I fibres were subclassified into A B and C subgroups (Brooke & Kaiser 1970 Dubowitz & Brooke 1973) The average number of counted fibres for classification into main groups (type I and type II) and subgroups (type I type II A B and C) was 585±5 and 531±35 respectively

The fibre areas were calculated from the lesser fibre diameter of each fibre type (type I and type II) using an eyepiece micrometer (Dubowitz & Brooke 1973). Assuming each fibre to have a circular cross-section with a diameter equal to the lesser fibre diameter the average cross-sectional area of each fibre type was calculated The fibre areas were determined from the NADH tetrazolium reductase staining (Novikoff et al 1961) and an average of 207±13 diameters were measured per subject The mean fibre area was calculated according to the formula presented by Häggmark et al (1978), i e fraction type I × type I fibre area + fraction type II × type II fibre area

Biochemical analyses Five enzymes were chosen to represent the major pathways in energy metabolism Glycolysis was represented by phosphofructokinase (PFK E C 2 7 1 11) lactate fermentation by lactate dehydrogenase (LDH E.C 1 1 1 27) fatty acid β-oxidation by *3-hydroxyacyl-CoA dehydrogenase* (HAD E C 1 1 1 35) the citric acid cycle by citrate synthase (CS E.C 4 1 3 7) and the respiratory chain by cytochrome oxidase (cytox E C 1 9.3 1) In addition two enzymes related to the contraction process and the short-term supply of ATP were investigated Mg^{2+}-stimulated ATPase (E C.3 6.1 4) and myokinase (MK E C 2 7 4 3). For PFK LDH HAD CS and cytox homogenates were prepared and assays performed as described previously (Örlander et al 1977) PFK was assayed according to Shonk & Boxer (1964) LDH and HAD by the methods of Bass et al (1969) CS as described by Srere (1969) and cytox according to Wharton et al (1969) For the assays of Mg^{2+} stimulated ATPase and MK the procedures described by Thorstensson (1976) were employed LDH isoenzymes were separated by disc-electrophoresis and relative quantities of M and H subunits were determined as described by Sjödin (1976) Protein was estimated by the method of Lowry et al (1951)

Electron microscopy Small pieces of muscle tissue were processed for electron microscopy and micrographs were taken for stereological analysis by methods described by Weibel (1969) An account of the procedure has been given before (Örlander et al 1977) It is of note that the values on mitochondrial mean volume and number per

nt volume are approximations (see Örlander et al 1977). The electron microscopical technique is very laborious as part of the investigation was limited to 26 subjects.

Functional measurements Maximum isometric and dynamic strengths were measured in the left knee-extensor muscles using an isokinetic dynamometer (Cybex II Lumex Inc New York) The subjects were seated in an adjustable chair with support for the back, shoulders and hips. The hip angle was fixed at π/2 rad (90°) and the lower leg moved the lever of the dynamometer The lever was set at constant length and the centre of the dynamometer axis of rotation was aligned with the anatomical axis of rotation of the knee joint. The angular movement of the knee joint was from 0.55π to 0 rad (100° to 0° i.e. full knee extension) The angular velocities studied were π/3 π/3 and π rad or 60° 120° and 180° In addition, comparisons were made with maximum isometric force measured with the same equipment with knee angles of π/6, π/3 and π/2 rad or 30° 60° and 90° The peak isometric value irrespective of knee angle was defined as the individual maximum isometric strength Two attempts were allowed at each knee angle and velocity and the highest values were noted The measurement were made in sequence from slow to fast speeds with 30 recovery between each contraction The following sequence was used π/6 rad, π/3 rad π/3 rad, 2 π/3 rad P rad and π rad High precision and accuracy for the torque registration have been reported by others (Moffroid et al 1969 Thorstensson et al 1976) The influence of muscular fatigue due to the testing procedure may be considered negligible (Torstensson 1976).

Muscular endurance was assessed in both isometric and dynamic terms Maximum isometric strength (MIS) and isometric endurance respectively were determined for both legs simultaneously (Karlsson & Ollander 1972) by recording the force exerted when the subjects pressed their feet against stiff bar equipped with force transducer MIS as taken as the highest force value obtained during series of 5 contractions After 3 min rest isometric endurance time was recorded, measured as the maximum time during which a tension level of 50% of MIS could be maintained

Dynamic endurance was measured as the ability to maintain tension output during repeated maximal dynamic contractions performed on the isokinetic dynamometer The angular velocity was pre-set at π rad (180°) with the subject seated as described above Dynamic endurance was calculated from measuring 50 maximal contractions and determining the relative (%) decline in peak torque from the mean of the three initial contractions, i.e. subjects with low force decline had high dynamic endurance and vice versa.

A more extensive presentation of the different functional measurements and the functional characteristics in relation to age is given elsewhere (Larsson & Karlsson 1978, Larsson et al 1979)

Statistics Intergroup differences were evaluated by means of Student' *t*-test, $P<0.05$ being accepted as significant. The possibility that mass significances (i false significances arising when comparing large numbers of parameters in limited experimental material) might occur was considered. However since the observed significant differences all seemed physiologically reasonable and coherent no restrictions were introduced on this ground.

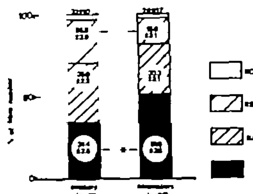


Fig 1 Fibre type distribution. Values are means ± S.E. denotes $P<0.01$

RESULTS

Fibre type distribution and fibre areas The proportion of type I fibres was significantly lower ($P<0.001$) in the smokers compared to the nonsmokers (38 1±3% $n=18$ and 50 7±5% $n=25$ respectively) Type II fibre subclassification revealed a correspondingly increased incidence ($P<0.01$) of type IIB fibres in the smokers (Fig. 1)

Fibre areas of each fibre type, fibre area ratios and mean fibre areas were almost equal in the two groups If anything, a tendency towards larger fibre areas was seen in the nonsmokers (Table 2)

Table 2 *Fibre areas*

Values are means ± S.E. n.s not significant

Group	No. of subjects	Type I area (μm^2)	Type II area (μm^2)	$\frac{\text{Type II}}{\text{Type I}}$ area ratio	Mean fibre area (μm^2)
Smokers	18	2 722±189	2 933±197	1 12±0 07	2 873±153
Nonsmokers	25	2 864±143	3 253 187	1 15+0.05	3 090±168
P			n.s	n.s	

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P		n.s	n s	n.s	n s	n s	n s	n s

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Statistics. Intergroup differences were evaluated by means of Student' ...t-test, $P<0.05$ being accepted as sig... ...nificant. The possibility that mass significances (i.e. false significances arising when comparing large numbers of parameters in a limited experimental material) might occur ...as considered. However, since the observed significant differences all seemed physiologically reasonable and coherent, no restrictions were introduced on this ground.

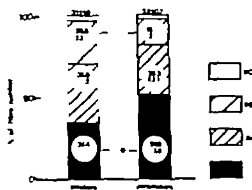


Fig. 1. Fibre type distribution. Values are means ± S.E. ... denotes $P<0.01$.

RESULTS

Fibre type distribution and fibre ...reas. The propor... ...tion of type I fibres was significantly lower ($P<0.001$) in the smokers compared to the nonsmokers (38.1±2.3% $n=18$ and 50.7±2.5% $n=25$ respectively). Type II fibre subclassification revealed a correspondingly increased incidence ($P<0.01$) of type II B fibres in the smokers (Fig. 1).

Fibre areas of each fibre type, fibre area ratios and mean fibre areas were almost equal in the two groups. If anything, a tendency towards larger fibre areas was seen in the nonsmokers (Table 2).

Table 2. *Fibre areas*

Values are means ± S.E. n.s. ...not significant

Group	No of subjects	Type I area (μm²)	Type II area (μm²)	Type II / Type I area ratio	Mean fibre area (μm²)
Smokers	18	2 722±189	2 933±192	1.12±0.07	2 873±153
Nonsmokers	25	2 864±143	3 253±187	1.15±0.05	3 050±168
P		n.s.		n.s.	n.s.

Table 1 *Anthropometrical characteristics*

Values are means ± S E FFS fat free soft tissue weight n s not significant

Group	No of subjects	Age (y)	Height (cm)	Weight (kg)	FFS (kg)	Skeletal weight (kg)	Body fat weight (kg)	Thigh circumference (cm)
Smokers	18	44±3	179±?	78 4± .6	53 9±1 7	1? 4±0.5	10 0±0 8	54 7±0 9
Nonsmokers	25	43±3	180±1	76 8±1 6	53 7±1.3	1 8±0.2	9.2±0 5	54 0 7
P		n s	n s	n s	n s	n.s.	n s	n s

MATERIAL AND METHODS

Subjects 43 apparently healthy men (18 smokers and 25 nonsmokers) volunteered to participate in the study They were all white-collar workers (employees of an insurance company) and engaged in little or no physical activity during their spare time The basis for acceptance of a subject was the desire to get a subject population as homogeneous as possible with regard to physical activity level Smoking habits were not considered in this selection. An oral consent was obtained from each subject after he had been informed of the procedure and possible risks of the experiments

Informations regarding smoking habits were obtained by using a questionnaire Each subject was asked to specify his weekly tobacco consumption 11 were cigarette smokers and smoked 91 (10–200) cigarettes with or without filters per week 4 subjects smoked the pipe and consumed 6 (25–125) g of tobacco per week 3 had "mixed" habits smoking both the pipe and cigars or cigarettes It was decided to consider all smokers together as one group

For each subject age height, weight skeletal width and skinfold thickness were recorded Body fat weight skeletal weight and fat free soft tissue weight (FFS) were calculated according to von Döbeln (1964) The circumference of the left thigh was measured in a horizontal plane just under the gluteal furrow As shown in Table 1 the two groups were very similar with regard to all studied anthropometrical variables.

All experiments were performed at 8–11 a m and followed a fixed protocol with biopsies preceding strength measurements No restrictions regarding smoking were given but no subject smoked during at least 20 min preceding the experiments

Muscle biopsies Muscle tissue was obtained from the left vastus lateralis by the percutaneous needle technique (Bergstrom 1962) The specimens were used for histochemistry enzyme assays and electron microscopy

Histochemistry The muscle samples for fibre typing and area determination were trimmed mounted frozen in isopentane cooled by liquid nitrogen and stored at −80°C until analysis

Serial transverse sections (10 μm) were cut with cryotome at −20°C and the myofibrillar ATPase staining method (Gomori 1941 Padykula & Herman 1955) was used for muscle fibre classification Photographs of the stained sections were taken and classification into type I (slow twitch) and type II (fast twitch) (Engel 1962) was made in all 43 subjects In 34 of the subjects, the type II fibres were subclassified into A B and C subtypes (Brooke & Kaiser 1970 Dubowitz & Brooke 1973) The average number of counted fibres for classification in main groups (type I and type II) and subgroups (type I type II A B and C) was 585±57 and 231±35 respectively

The fibre areas were calculated from the "lesser fibre diameter" of each fibre type (type I and type II) using an eyepiece micrometer (Dubowitz & Brooke 1973) Assuming each fibre to have a circular cross-section with a diameter equal to the "lesser fibre diameter" the mean cross-sectional area of each fibre type was calculated The fibre areas were determined from the NADH tetrazolium reductase staining (Novikoff et al 1961) and an average of 207±13 diameters were measured per subject The mean fibre area was calculated according to the formula presented by Haggmark et al (1978) i e fraction type I × type I fibre area + fraction type II × type II fibre area

Biochemical analyses Five enzymes were chosen to represent the major pathways in energy metabolism Glycolysis was represented by phosphofructokinase (PFK E C 2 7 1 11) lactate fermentation by lactate dehydrogenase (LDH E C 1 1 1.27) fatty acid β-oxidation by 3-hydroxyacyl-CoA dehydrogenase (HAD E.C 1 1 1 35) the citric acid cycle by citrate synthase (CS E C 4.1 3 7) and the respiratory chain by cytochrome oxidase (cytox E C 1 9.3 1) In addition two enzymes related to the contraction process and the short-term supply of ATP were investigated, Mg^{2+} stimulated ATPase (E C.3 6.1 4) and myokinase (MK E C.2 7 4 3) For PFK LDH HAD, CS and cytox homogenates were prepared and assays performed as described previously (Örlander et al 1977) PFK was assayed according to Shonk & Boxer (1964), LDH and HAD by the methods of Bass et al (1969), CS as described by Srere (1969) and cytox according to Wharton et al (1969) For the assays of Mg^{2+} stimulated ATPase and MK the procedures described by Thorstensson (1976) were employed LDH isoenzymes were separated by disc-electrophoresis and relative quantities of M and H subunits were determined as described by Sjödin (1976) Protein was estimated by the method of Lowry et al (1951)

Electron microscopy Small pieces of muscle tissue were processed for electron microscopy and micrographs were taken for stereological analysis by methods described by Weibel (1969) An account of the procedure has been given before (Örlander et al 1977) It is of note that the values on mitochondrial mean volume and number pe

able 5 *Isometric nd dynamic strengths as ell as dynamic strength in percent of isometric strength at fferent ngular elocities*

alues are means S.E. s., not significant

Group		Isometric strength 0° (Nm)	Dynamic strength 60° Nm	%	120° Nm	%	180° Nm	%
Smokers	18	180 9±9.5	165 9_8 4	93 0±0 03	143 0±5 4	80 9±0 03	1 1 7±7.0	68.5±0 04
Nonsmokers	25	216.5+6 6	194.2±6 0	90.3_0 02	16_6_5 1	75.5±0 02	131.0 4 3	60 8±0 01
		<0 01	<0 01	n s.	<0.05	s.	s.	<0 05

Muscular endurance measured both in short erm isometric and dynamic endurance tests was not significantly different in the two groups (Table 6).

DISCUSSION

The present investigation has shown some quite clear-cut differences in skeletal muscle morphology, metabolism and function between smoking and nonsmoking sedentary men. Significant reductions in muscle oxidative capacity and strength were seen in the smokers, effects which may be partly related to differences in fibre type distribution.

Fibre type distribution

It is evident that the lower proportion of type I fibres and the concomitantly higher percentage type IIB fibres in the smokers (Fig 1) may either be explained as an effect of smoking *per se* or it may be that some background factor is responsible. Both these possibilities will be considered.

There is ample evidence that the "type" characteristics of a muscle fibre are controlled by the motor nerve through "trophic" influences (Guth 1968, Close 1972, Gutmann 1976). Recent evidence indicates that the control may be purely a function of impulse activity (Salmons & Sréter 1976). While human type II A and II B fibres seem to some extent interconvertible by intense training (Andersen & Henriksson 1977, Jansson & Kaijser 1977), it appears that type I–type II interconversions in adult muscle can be achieved only by means such as cross innervation or electrical stimulation (Guth 1968, Salmons & Vrbová 1969, Close 1972, Pette et al. 1973). It is thus hard to envisage a mechanism whereby tobacco smoking might produce such an alteration. Nicotine would be a conceivable etiological agent, since it stimulates muscle fibres at the motor endplate and interferes with acetylcholine metabolism (Fischer et al. 1960, Larson et al. 1961). A disturbance of the normal neuromuscular interaction by such effects might possibly alter the fibre type distribution pattern.

Negative correlations have been found between the amount of smoking on the one hand and indices of physical fitness and sports interest on the other (Bremner et al. 1977). In a study of 16-year-old boys and girls, Hedberg & Jansson (1976) found that those with a low percentage type I fibres in vastus lateralis were less active and less interested in physical activities than those with a high percentage. By integrating these observations one might suggest that those with a low proportion type I fibres are more liable to smoke; this would provide an alternative explanation for the present finding. There is evidence for a strong genetic influence on fibre type distribution in man (Komi et al. 1977). It

Table 6. *Isometric and dynamic endurance*

Values are means ± S.E. Numbers of subjects within brackets. MIS maximum isometric strength. n. not significant.

Group	Isometric endurance at 50% MIS (s)	Dynamic endurance Decline in peak torque, 50 contr 180° (%)
Smokers	51 9±4.8 (18)	50 0±2.9 (12)
Nonsmokers	65 2±6 9 (25)	49 0±1 1 (21)
P		n.

Recent observations by Jansson et al. (1978) do, however, suggest that extreme training might produce type I–type II interconversion.

Table 3 *Enzyme activities*

Values are means ± S E For full names of enzymes see Material and methods n.s not significant

Group	n	PFK	LDH	HAD	CS	Cytox μmol O_2 × min × (g wet wt)⁻	Mg² ATPase	MK	H-LDH % of LDH subunits
		μmol × min × (g wet wt)					μmol × min × (g wet wt)		
Smokers	18	8 13 ±1 16	71 1 ±7 0	1 77 ±0 10	3 40 ±0 20	3.26 ±0.32	11 1 ±1 0	150 7[b] ±11 4	30 6[b] ±3 7
Non smokers	25	8.56 ±0 73	75 1 ±6 4	2.25 ±0.14	4.53 ±0.22	4 26 ±0 28	10 9[a] ±0 7	177.9[a] ±7 3	34 6[c] +
P		n.s	n.s	<0 05	<0 001	<0 05	n s	n s	n s

[a] 24 subjects [b] 16 subjects [c] 23 subjects

Enzyme activities The activities of the 7 studied enzymes in the two groups are shown in Table 3

PFK LDH (including subunit percentages) Mg² stimulated ATPase and MK were not significantly different in the two groups For the mitochondrial enzymes significantly lower (HAD 21% $P<0$ 05 CS 25% $P<0$ 001 cytox 23% $P<0$ 05) activities were found in the smokers No difference in muscle protein content was observed

Ultrastructure Table 4 gives the values on mitochondrial volume fractions as well as apparent numbers and mean volumes in the fibrillar and subsarcolemma spaces of muscle fibres The volume fraction of lipid droplets is also indicated

The only significant intergroup difference was a diminished fibrillar space mitochondrial volume fraction (21% $P<0$ 01) in the smokers A similar trend could be seen for the corresponding mean volume (23% lower in smokers) this difference was however not statistically significant ($P<0$ 2)

No significant difference in lipid droplet content was present but when those smokers who smoked 50 g of tobacco or more per week were considered separately a significantly ($P<0$ 05) reduced value was observed (0.35±0 08% $n=5$)

Muscle strength and endurance are shown in Tables 5 and 6 Maximum isometric strength measured either as a one or a twoleg contraction was found to be higher ($P<0$ 05–0 01) in the non smokers (*Table 5*) *This also held true for dynamic* strengths ($P<0$ 05–0 01) except for the highest angular velocity studied (π rad × s or 180° × s⁻¹) which was almost equal in the two groups (Table 5) When dynamic strengths were expressed in relation to maximum isometric strength it was found that the smokers were relatively stronger at the highest angular velocity (π rad × s) whereas no statistically significant difference was seen at the other velocities studied (Table 5) This seems to indicate a quantitative as well as qualitative difference in muscular strength between the two groups

Table 4 *Ultrastructural parameters*

Values are means ± S E Note that numbers and mean volumes are approximations (see Material and methods) n s not significant

Group	n	Mitochondria in fibrillar space: Volume fraction (%)	Number μm⁻³	Number Sarcomere	10⁴ × Mean volume (μm³)	Mitochondria in subsarcolemma space: Volume fraction (%)	Number (μm)	10³ Mean volume (μm³)	Lipid droplet Volume fraction (%)
Smokers	14	3 79 ±0 [illegible]	1 43 ±0 17	1 03 ±0 10	3 63 ±0 40	27 6 ±3 6	5 34 ±0.58	6 18 ±1 09	0 70 ±0 15
Non smokers	12	4 79 ±0 28	1 35 ±0 1	1 19 ±0 10	4 70 ±0.58	23 9 ±3 3	4 68 ±0 96	7 1 ±1 93	0 97 0 15
P		<0 01	n.s	n s	n s.	n s	n.s	n	n s

13 subjects

...earch Council (B77-04X-4251-04A, B78-04X-4251 ...B), the Research Council of the Swedish Sport Federation, Svenska sällskapet för medicinsk forskning, Skandia Insurance Company and the Swedish Tobacco Company

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would be tempting to speculate that there exists a hereditary predisposition towards taking up smoking this might be related to the well-established connection between smoking behaviour and personality traits (Cherry & Kiernan 1976 Simon & Primavera 1976) Any conclusions on this point would however be premature at the present time

Muscle metabolism

The enzyme activity determinations revealed a lower muscle oxidative capacity in the smokers HAD CS and cytox activities being 21–25 % lower than in the nonsmokers (Table 3) Ultrastructurally this was reflected by the equally decreased (21 %) fibrillar space mitochondrial volume fraction (Table 4) A lower mean volume per mitochondrion appeared to be the main cause for the decreased volume fraction even though this difference failed to reach statistical significance

The impaired oxidative capacity in the smokers muscles may be due to various causes Essén et al (1975) found proportions of 2 5 1 9 1 2 4 for succinate dehydrogenase activity in type I IIA IIB and II C fibres respectively By using these values and those in Fig 1 it can be estimated that some 40–50 % of the difference in oxidative capacity may be attributed to the difference in fibre type distribution A lower physical activity level in the smokers would contribute to the lowered oxidative capacity (cf Holloszy & Booth 1976) The fact that subjects were recruited on the basis of a low and homogeneous occupational and sparetime physical activity level and the very small differences in anthropometrical characteristics (Table 1) tend to dispute this possibility The similarities in fibre areas (Table 2) point in the same direction Furthermore smokers and nonsmokers reported similar sparetime activities in a questionnaire It can however not be ruled out that the smokers might have adapted to a somewhat more sedentary way of life since there are indications that smokers generally tend to dislike strenuous physical activities (Biersner et al 1977) Finally it is conceivable that some of the well over 1200 substances found in tobacco leaf and/or smoke (Stedman 1968) might influence enzyme levels in muscle For example carbon monoxide has been suggested to produce tissue hypoxia (e g Klausen et al 1968 Sagone et al 1973) which might depress the synthesis of mitochondrial enzymes (e g Hakami & Pious 1967 Simon & Robin 1970 Hallman 1971 Mela et al 1976 Kinnula 1976)

Functional characteristics

The most striking finding in the functional characteristics of the muscle was the lower muscle strength in the smokers expressed in isometric as well as dynamic terms The relative difference amounted to approximately 16 % for isometric strength and 15, 12 and 7 % for dynamic strength at the different angular velocities corresponding to 60, 120 and 180° × s^{-1} respectively

It should be noted that the absolute and relative difference in maximum strength between the two groups decreased with increasing speed of contraction (Table 5) At the highest speed of contraction this difference was not statistically significant. This is not surprising since the smokers had a higher proportion of type II muscle fibres which is known to be essential to a high force output during fast muscle contractions (cf Burke & Edgerton 1975). This is most likely due to the short contraction time of type II fibres (Close 1964) In fact when dynamic strengths were expressed as percent of maximum isometric strength a significantly higher value was found for smokers at the highest angular velocity (Table 5) This is in accordance with Thorstensson et al (1976) who demonstrated a positive correlation between percent type II fibres and dynamic strength at 180° × s expressed as percent of maximum isometric strength

The reason for the lower absolute muscular strength in the smokers is at present not known Muscular strength is mainly dependent upon the cross sectional area of the muscle and the number of recruited muscle fibres Since neither thigh circumference (Table 1) nor fibre areas (Table 2) differed between the two groups a different cross-sectional area does not seem very plausible A different muscle fibre recruitment pattern thus appears to be the most likely cause for the lower muscular strength in the smoking subjects It is however too early to speculate upon the mechanism for this, and further studies will be undertaken specifically to analyze these questions

We are indebted to Mrs Gun-Britt Örlander and Mrs Maria Ahlberg for skilful technical assistance and to Mrs Carina Erikson for secretarial assistance Financial support has been received from Magn Bergvall Foundation Clas Groschinsky Memorial Fund the Faculty of Science of the University of Uppsala, the Swedish Medical

esearch Council (B77-04X-4251-04A B78-04X-4251 -9B), the Research Council of the Swedish Sport Federation, Svenska sällskapet för medicinsk forskning, Skandia Insurance Company and the Swedish Tobacco Company

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plantar flexion strength and structure

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FUGL MEYER A. R. SJÖSTRÖM M & WÅHLBY L. Human plantar flexion strength and structure. Acta Physiol Scand 1979 107 47 56. Received 25 May 1978 ISSN 0001 6772. Department of Physical Medicine and Rehabilitation Departments of Anatomy and Neurology and Department of Surgery University of Umeå S eden

Plantar flexion strengths were studied in 30 right-handed males Static and dynamic maximum plantar flexion torques ere recorded, knees fully extended (0°) and in 90° flexion In five of the subjects the soleus and gastrocnemius muscle structure were studied by light microscopy and enzyme histochemistry Specimens were obtained by needle biopsy usually bilaterally Intraindividual declines of force were found to be function of angular motion velocity Static and dynamic torques correlated significantly Peak torques were significantly greater (mean 15%) at the 0° than at the 90° knee angle and left maximum plantar flexion torques at 0° were greater (mean 10%) than right. Mean morphometric data on the m soleus suggested right-left ymmetry which could not be demonstrated for the m. gastrocnemius. Fibres ith low stainability for myofibrillar ATPase (Type 1 fibres) had smaller diameters, but constituted the major part of the cross-sectional areas I these five non-athletes no significant correlation between data on plantar flexion trength and morphometric data on triceps surae structure could be demonstrated On the other hand, strength covariated with calf circumference.

Key ords Human, skeletal muscle needle biopsy morphology muscle fibres, physiology strength, isokinetic isometric biomechanics

This investigation was designed to study the plantar flexion strength characteristics in younger adult males and to analyze whether structural details of the triceps surae muscle can predict plantar flexion strength in normal non-specifically trained subject Theoretically the triceps surae accounts for about 90% of the maximum plantar flexion strength in man (Murray et al 1976) While the length tension relationships of the gastrocnemius muscle can be passively altered by changes of knee position such is not the case for the one-joint soleus muscle This latter part of the triceps surae is of primary importance in stabilizing the talocrural joint when in the upright position (Granit 1970 Campbell et al 1973)

Human triceps surae muscle contains fibres that can be differentiated on the basis of myofibrillar adenosine triphosphatase activity aerobic and anaerobic potential (Edström & Nyström 1969 Johnson et al 1973 Costill et al 1974 1976 Gollnick et al. 1974 Taylor et al. 1974 Edgerton et al. 1975) However these reports do not include data on fibre size and relative occurrence of the histochemically differentiated fibre categories (cf Table 4) Thus hitherto it has not been feasible to estimate the relative cross-sectional fibre area, and to correlate this to the physiological cross-section (Fick 1911) Furthermore in the field of occupational ergonomics, it is of interest to analyze wheter morphological asymmetry occurs in untrained man

Until recently the methods available for clinical assessment of plantar flexion strength have mostly applied measurements of static strength. Generally semi-objective functional measures (e g. standing on toes, climbing steps) or strength measurements in standardized positions have been applied (Kendall & Kendall 1969 Daniels & Worthingham 1972) Such methods have, however been criticized as being too indiscriminative (Herman & Bragin 1967) Objective strength measurements using electronic

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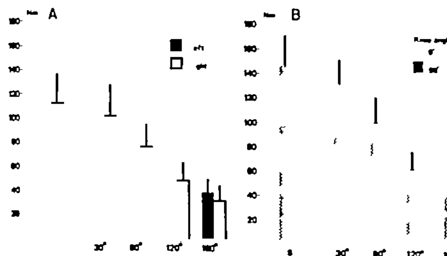


Fig 3 Relation in 5 healthy sedentary adult males between mean maximum plantar flexion torque of (A) the right and the left legs with knees flexed 90° and (B) the left leg with knee straight (0°) and rigidly flexed (90°). Torque ± S D (Nm) is given as function of angular velocity *S* indicates static manoeuvre

be torque was measured with the foot-plate locked in the angular position identical to the peak torque position for the slow (30°/s) dynamic manoeuver This angular position was chosen for direct comparison of the static with the dynamic forces.

Morphological studies 1 5 of the subjects 3 separate muscle biopsies were performed by the same surgeon after strength measurements. The biopsies were taken from both soleus muscles approximately 17 cm above the calcaneus and from the maximum bulge of the lateral gastrocnemius using the needle biopsy technique (Bergström 1975) The samples were frozen by immersion in Freon 12 chilled by liquid nitrogen. Serial cross-sections, ten mic ron thick, were cut in cryostat at 20°C and mounted on glass slides. The specimens were then treated histochemically for myofibrillar adenosine triphosphatase (ATPase) at pH 9.4 4 6 and 4.2 and succinic dehydrogenase (SDH) and with haematoxylin-eosine (Dubowitz & Brooke 1973).

RESULTS

Strength. The mean maximum static torques in both positions were greater than the peak dynamic torques at 30°/s (Table 1) The relationship between mean maximum plantar flexion torque of the right and left legs is displayed in Fig. 3*a* With increasing angular velocity of motion the peak dynamic torques decreased Static and dynamic peak torques (Table 1) covariated significantly ($P < 0.001$ Pearson correlation) (Table 2) for both legs and for both knee positions. Mean maximum static and dynamic plantar flexion torques in both positions were significantly greater ($P < 0.05$ Student's *t*-test for paired observations) for the left than for the right legs. Furthermore plantar flexion strength was significantly greater ($P < 0.05$ Student's *t*-test for paired observations) in the 0° than in the 90° knee position (Fig. 3*b*) Virtually identical plantar flexion strength measurements with the knees in the 90° position were obtained irrelevant of hip position Dynamic plantar flexion strength at 30°/s straight leg correlated significantly (right 0.59 left 0.49- $P < 0.001$) with calf circumference In the five subjects studied morphologically right/left strength differences were systematically as pronounced as for the total sample studied with the exception of 30°/s manoeuvers (cf Table 1) The mean strength difference in maximum plantar flexion strength for the two knee positions were, however nearly identical for the *total sample* and the subsample The plantar flexion strength in the static and in the slow dynamic manoeuvres was about 15–20% and in the two fastest dynamic manoeuvres about 10–15% lower for the 90° than for the 0° knee position. The range of maximum torques was greater for manoeuvres performed with flexed than for those performed with extended knees (cf Table 1 ± S.D)

Structure The muscle biopsies of which two from the gastrocnemius had to be omitted due to technical failure showed polygonally packed muscle fibres of equal size (cf Table 3) interspersed with sparse connective tissue (Figs 4 and 5) A few small rounded muscle fibres were found and one or

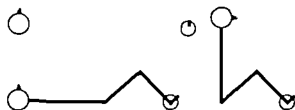


Fig 1 Testpositions for measurements of static and dynamic plantar flexion strength In left drawings subjects are placed supine to the right upright sitting.

equipment have also been described (cf Darcus 1953 Asmussen & Heeboll Nielsen 1961 Tornvall 1963)

Since the functional demands on plantar flexors are dynamic in character even in their postural alignment function dynamic force should be the most adequate measurement for their characterization strengthwise The Cybex II (Lumex New York) dynamometer offers possibilities of measuring both the maximum static (isometric) and the dynamic forces For the latter measurements the concept of isokinetics as first described by Hislop & Perrine (1967) and Thistle et al (1967) is applied This concept provides opportunities of measuring the dynamic force at any constant predetermined angular velocity of motion greater than zero and up to 300 degrees per second The validity and reliability of the device has been discussed by Moffroid et al (1969)

MATERIAL AND METHODS

30 clinically healthy right-handed men (mean age 30 1 years S D 5 7) were investigated for static and dynamic (isokinetic) plantar flexion strength None of the subjects were engaged in regular physical training and all had sedentary occupations. Calf circumference measured in the standing position at the horizontal intersection between the upper and the middle thirds of the lower leg, was 36.9 cm S D 2, for the right and 36 6 cm S D 2 for the left leg. In 5 of the men (mean age 31 4 years S D 4.5) morphological studies were also performed

Strength measurement For measurements of strength two Cybex II dynamometers (Lumex New York) were used one for each leg This instrument allows for linear torque measurements from 10 to a maximum of approximately 550 Newtonmetres (Nm) within 1% of the latter maximum registrable torque (Moffroid et al 1969) Simultaneous registrations of torque and angular motion of the ankle joint were displayed on an X Y storage-oscilloscope (Tektronix Oregon) and registered on an ink writer with an adequate linear frequency response of 3 dB at 1200 Hz (Mingograph Siemens Elema, Sweden) The recording instruments were coupled in parallel.

The subjects were placed in the supine position on a plinth Two leg positions were applied Either the hips and knees were in the 0° position or knees were in the 90° flexed position and the hips approximately 45° flexed (Fig 1) In the 0° position the knees and hips were fixed to the plinth by velcro-bands. In the 90° knee position a padded right angled board was fixed firmly with velcro-band to the dorsal surface of the leg extending 40 cm above and below the knee thus securing a rigidly standardized 90° knee-angle In another series of experiments subjects were seated (Fig. 1) with hips virtually maximally flexed and knees 90° flexed The feet were firmly strapped to the Cybex II foot plate and care was taken to align the flexion extension axis of the talo-crural joint with that of the apparatus

Each subject was instructed to perform maximum isokinetic plantar flexions of the ankle joint at preset angular motion velocities of 30° 60° 120° and 180°/s starting at maximum dorsi-flexion Care was taken that the subject dorsi-flexed slowly pausing shortly in the maximum dorsi-flexed position The manoeuvres were displayed on the X–Y oscilloscope with force registered on the Y-axis and angle on the X-axis (Fig) This was done to assertain that the subjects were familiar with the experimental procedure and able to produce virtually similar maximum force-angular curves throughout the range of motion thus validating the procedure The maximum sta

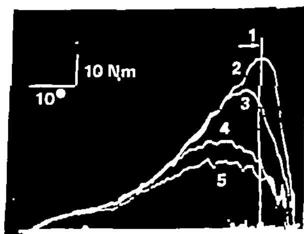


Fig 2 An example of an X Y registration of static and dynamic plantar flexion torques Torque is displayed on the Y-axis and angle on the X-axis Maximum dorsal flexion is to the extreme right and maximum plantar flexion to the extreme left (1) denotes maximum static torque in the angular position identical to the peak torque position for the slow (30°/s) dynamic manoeuvre It was here used for direct comparison of static and dynamic forces. ( ) denotes maximum dynamic torque throughout the range of motion of the ankle joint at a preset velocity of 30°/s (3 4, 5) denote torque at angular velocities of 60°/ 120°/s and 180°/ respectively

L Nm	90° R %	L %	180°/s 0° R Nm	L Nm	90° R %	L %
69	98	101	40	49	108	80
95	83	82	51	[illegible]	67	65
59	97	83	36	57	100	86
72	96	79	37	48	111	97
68	70	67	30	35	83	86
71	89	82	39	46	94	87
13	13	15	8	11	7	6
63	86	90	36	42	89	93
14	15	14	9	12	12	11

Table 2. Coefficient of correlation between maximum static and dynamic peak torques in right (upper figure) and left (lower figure) leg of 30 clinically healthy subjects between 20 and 39 years

Measurements performed in the supine position with knees fully extended (0°)

	30°/s	60°/s	120°/s	180°/s
Static	0.76 0.93	0.85 0.86	0.63 0.76	0.59 0.70
30°/s		0.84 0.87	0.78 0.81	0.73 0.77
60°/s			0.88 0.81	0.85 0.72
120°/s				0.94 0.88

cal comparisons of intraindividual structural side differences within the m. gastrocnemius were not performed on account of the small number of bilateral biopsies. In contrast to the tendency towards mean bilateral structural symmetry considerable intraindividual side differences in both fibre composition and diameter were found in several cases exceeding 10%. This was especially noticeable for the fibre composition and fibre diameters for subject B (cf. Table 3). Evidently due to these discrepancies the relative cross-sectional areas also varied. The product of the fibre cross-sectional area and the physiological cross-section was calculated for the total triceps surae and for its separate components (Table 3). According to Fick (1911) the mean physiological cross-sectional area of the triceps surae is 43 cm² of which the m. soleus represents 47% and the m. gastrocnemius 53%. However the product of the relative morphological and the physiological cross-sectional areas also varied widely intra- and inter-individually. For the latter calculations it was assumed (cf. Johnson et al. 1973) that the biopsy specimens obtained from the deep fascicles of the lateral head of the gastrocnemius muscle possess a structure identical to that of the medial head.



Fig. 5. Serial section of m. gastrocnemius treated for myofibrillar ATPase at pH 9.4, at pH 4.6 and for succinyl dehydrogenase. At pH 9.4 the amount of heavily stained fibres are almost equal of lightly stained fibres. At pH 4.6 the heavily stained fibres show both intermediately and lightly staining characteristics. Also at the SDH staining 3 different fibre types could be differentiated. ×100

Table 1 *Individual static and dynamic plantar flexion peak torques for the right (R) and left (L) kn[ee] healthy sedentary males*

	Static				30°/s				60°/s			
	0°		90°		0°		90°		0°		90°	
	R Nm	L Nm	R %	L %	R Nm	L Nm	R %	L %	R Nm	L Nm	R %	L
A	163	159	71	84	141	130	80	85	108	104	97	[illegible]
B	180	191	84	77	162	158	93	80	[illegible]	138	[illegible]	[illegible]
C	179	123	87	91	119	114	85	86	85	96	91	[illegible]
D	139	150	81	86	179	135	84	84	96	108	89	[illegible]
E	125	140	100	64	105	115	81	74	90	90	[illegible]	[illegible]
$\bar{X}$	147	153	85	80	131	130	85	82	101	107	84	[illegible]
±S D (Nm)	24	25	17	[illegible]	22	18	24	16	16	19	15	16
$\bar{X}$ (n=30)	139	146	88	84	123	132	84	85	91	101	85	[illegible]
±S D (Nm)	[illegible]	[illegible]	4	[illegible]	[illegible]	19	[illegible]	[illegible]	19	[illegible]	18	16

Mean values + S D for these and for additionally 25 healthy males are given Measurements were performed in the [...] position knees either fully extended (0°) or flexed (90°) Values for the 90° position expressed as per cent of the correspo[nding] value at 0° S D calculated from actual performance (Nm)

two internal nuclei were occasionally found No diffuse or frequent focal abnormality was seen In sections treated for myofibrillar ATPase at pH 9 4 fibres with low stainability generally dominated In the m soleus scattered intensively stained fibres were also seen (Table 3 Fig 4*a*) while in some specimens from the m gastrocnemius such high ATPase fibres were more abundant (Fig 5*a*) In sections treated for myofibrillar ATPase but pre-incubated at pH 4 2 and 4 6 the picture was reversed In the m soleus at pH 4 2 less than 1% showed intermediate staining characteristics (Fig 4*b*) while although within a wide range (1 [illegible]) such fibres occurred more frequently in the lateral gastrocnemius muscle at pH 4 6 (Fig 5*b*) High SDH intensity corresponded to fibres with low stainability for ATPase at pH 9 4 and vice versa In the m soleus these categories showed only subtle variations in SDH stainability and subclassing was considered invalid (Fig 4*c*) For the m gastrocnemius such subclassing could be performed (Fig 5*c*).

In the m soleus there was no significant left/right asymmetry regarding mean fibre diameter [for] type (Table 3) or fibre cross sectional area. Stainab[...]

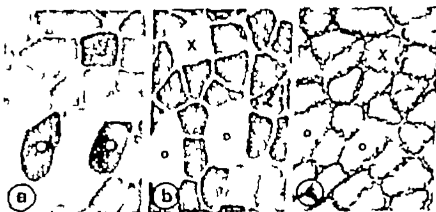


Fig 4 Serial sections of m soleus treated for myofibrillar ATPase at pH 9 4 at pH 4_ and for succinic dehydrogenase At pH 9 4 a dominance of fibres with low stainabil ty is seen with scattered highly stained fibres (and *o*) After preincubation at pH 4_ a few (less that 1% of total) of these latter fibres (*x*) showed intermediate taining characteristics ×100

h			180°/s			
	90°		0°		90°	
L Nm	R %	L %	R Nm	L Nm	R %	L %
68	98	101	40	50	108	80
95	83	82	51	[illegible]	67	63
59	97	83	36	37	100	86
77	96	79	37	48	111	92
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120°/				0.94 0.88

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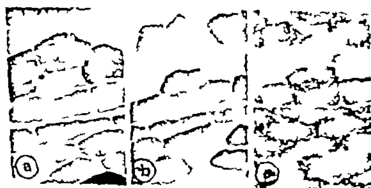


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Table 3 *Morphometric results on the triceps surae muscle*

Muscle fibre appearance and fibre diameter were determined on the basis of low and high stainability in sections tre for myofibrillar ATPase at pH 9 4 R = right L = left

Subject	A		B		C		D		E		Mean ± S D	
Leg	R	L	R	L	R	L	R	L	R	L	R	L
Fibre (%)												
Low ATPase												
M soleus	7?	69	77	70	70	81	74	82	83	84	75±5	77+7
M gastrocnemius	56	7?	46	87	67	81	–	87	79	–	–	
Fibre diameter (μm)[a]												
Low ATPase												
M soleus	70	77	71	79	71	68	6.	73	83	80	71±8	75±5
M gastrocnemius	57	63	61	71	62	65	–	59	64	–	–	
High ATPase												
M soleus	82	90	80	85	83	75	66	75	9?	9	81±9	83+8
M gastrocnemius	58	64	64	80	68	65	–	77	81	–	–	
Product of relative fibre cross-sectional area and physiological cross section												
Low ATPase												
M soleus	1 304	1 511	1 056	1 612	1 304	1 382	1 049	1 61	105	1 985	1 364±433	1 630 2?
M gastrocnemius	757	1 188	713	1 825	1 071	1 426	–	826	1 348	–	–	
High ATPase												
M soleus	694	9?7	543	801	763	393	418	374	531	900	590±138	599+2?
M gastrocnemius	616	478	939	314	609	503	–	1 118	41	–	–	

All fibres per biopsy were counted (usually more than 1 000)
[a]One hundred fibres of each type were measured according to Schmitt (1976)
(Fibre diameter/?)$^2\pi$ × relative occurrence × percent of the total physiological cross-section according to Fick (1911)

Spearman rank correlation calculations (r) were performed on the series of ten legs (soleus) and on the eight legs (gastrocnemius) to elucidate whether the structural findings of either the soleus or the gastrocnemius or the combination of these muscles were correlated to maximum torques in static and dynamic manoeuvres However no significant strength–structure correlation ($P<0\ 05$) was obtained

DISCUSSION

Strength

Normative data on static and dynamic strengths for left and right plantar flexion in healthy sedentary younger Swedish males between 20–39 years are presented To our knowledge there are no previous descriptions of dynamic plantar flexion strengths presented elsewhere A relationship between plantar flexion strength and calf circumference is demonstrated here Furthermore because of the design of the experimental procedures measurements were monitored (see Methods) and left and right plantar flexion strengths were tested alternately first we feel that the intraindividual right/left strength differences were real

The significant co-variance between static and dynamic strength in both legs or normals corroborates the findings of Asmussen et al (1965) in the upper extremity However in the triceps brachii Osternig et al (1977) could find only randomized correlations between isokinetic and isometric torques The maximum dynamic strength of the human plantar flexor muscles in the untrained males was characterized by a decline at higher angular motion velocities This finding is in good agreement with that of Thorstensson (1976) in the quadriceps muscle An interesting deduction is that "non-functional" measurements of static strength are also representative of dynamic manoeuvers at least in untrained adult males When measuring maximum plantar flexion torques it seems most appropriate to perform measurements with knee extended as in this position length-tension relationships of all parts of the triceps surae are optimal for development of

aximum force (Herman & Bragin 1967). In the present series of expts. hip position did not seem to influence plantar flexion strength.

We can offer no obvious explanation for the significant dominance of the left leg plantar flexion strength over the right leg. Coincidence can however be excluded as in a greater series of male and female subjects between 20 and 65 ($n=134$) we found a significant dominance of left plantar flexion at identical levels (Fugl-Meyer et al. to be published). The relationship between calf circumference and strength is in agreement with the findings of Lamphiear & Montoye (1976) and O'Donovan & Watson (1977) who propose that the maximum strength of muscles in the upper extremities is correlated to different anthropometrical variables.

It is reasonable to assume that of the two chosen positions strength is greater in the 0° than in the 90° knee position. In the former of these positions length-tension relationships are optimal for both the one-joint m. soleus and the two-joint m. gastrocnemius. In another study of 5 young men (Fugl-Meyer, Barstedt & Långström in manuscript) we additionally registered the rectified low-pass filtered electromyogram from all three parts of the triceps surae using surface electrodes. The peak tension of the m. soleus was identical for manoeuvres performed in the 0° and in the 90° knee position. In contrast for both heads of the gastrocnemius the peak tension at the 90° position was significantly lower ($P<0.001$) for the static and for each dynamic manoeuvre. The decrease averaged about 45%. Furthermore in the straight knee position for all the different manoeuvres the peak activation of the three parts of the triceps surae did not differ significantly. In the 90° knee position the m. soleus behaved in the same manner. In this position both heads of the gastrocnemius had higher peak tensions at the isokinetic than at the static manoeuvres. This phenomenon may explain the smaller peak torque differences between the 0° and 90° knee position at higher angular velocities.

In younger males the triceps surae constitutes about 70% of the total plantar flexor weight and volume (A. Eriksson et al. unpublished observations). The physiologic cross-section is, according to Fick (1911) of an identical magnitude. As the lever arm of the triceps surae is about double the length of that of the remaining plantar flexors (Jergeson quoted in Murray et al. (1976)) the triceps surae is by far the dominating plantar flexor. Thus Fick (1911) and Murray et al. (1976) calculated that in the straight leg position the m. gastrocnemius would contribute about 45% and the m. soleus about 35–40% to the maximum plantar flexion torque.

The lower peak torques in the 90° as compared to the 0° knee position, averaging about 80–90%, can thus be explained by the smaller peak activity of the m. gastrocnemius in the 90° position. In the straight knee position the m. soleus is therefore the dominant plantar flexor contributing at least 50% to the maximum plantar flexion torque.

Structure

The morphometric results from the human triceps surae described here are fairly similar to findings previously presented elsewhere (Table 4). This conclusion is obviously made on the assumption that the fibres by different authors termed as "red", "slow-twitch" and "Type I" fibres all correspond to the fibres termed here as "ATPase low at pH 9.4". We have chosen this terminology simply due to the fact that our only criterium (which is also used by all the other authors listed in Table 4) for classifying the fibres is the degree of stainability in thawed thin cryosections for myofibrillar ATPase at pH 9.4. This classification of muscle fibres is also frequently used in clinical diagnostic work (Dubowitz & Brooke 1973, Engel 1974). It has been pointed out elsewhere (e.g. Edgerton et al. 1975) that myofibrillar ATPase reflects at least one very useful functional parameter, namely the speed of contraction. Direct proofs for this correlation in the soleus muscle of the rat have been presented by Kugelberg & Edström (1968) and Kugelberg (1973). However, no such evidence exists concerning the human triceps surae. Furthermore, there are morphological differences between the rat and the human soleus muscle both at light microscopical level, i.e. the degree of stainability for SDH and distribution of SDH stain precipitate (Edgerton & Simpson 1969) and at electron microscopical level e.g. concerning the myofibrillar M-band (Sjöström & Squire 1977 (rat); Sjöström, unpublished observations (human soleus)). Comparisons between different species, even when concerning the same muscle should therefore be made with great caution.

Johnson et al. (1973) found only minor differences between the deep and superficial portions of the human soleus muscle regarding the occurrence of Types 1 and 2 fibres. This seems to be analogous with the statements by Gollnick et al. (1972) and

Table 4 *Fibre composition of the human triceps surae in different studies*

Source	Age Mean	Age Range	Biopsy technique	Nomenclature	Fibre occurrence and ... M soleus %	M soleus Range ± S D
Edström-Nyström (1969)*	–	–	Needle	Red	71	65–78
Johnson et al (1973)	–	17–30	Autopsy	Type I	86 deep 89 superf	70–100 77–100
Costill et al (1974 1976)	25	23–28	Needle	*Slow twitch*	83	
Gollnick et al (1974)	–	1–41	Needle or surgical	Slow twitch	80	64–100
Taylor et al (1974)	Adult	–	Needle	Slow twitch	77	
Edgerton et al (1975)	59	1–83	Autopsy	Slow twitch	70	18
Present material	31	4–36	Needle	ATPase low	79	69–91

* Bilateral study

Edgerton et al (1975) which were based on findings from the vastus lateralis muscle that the proportion of slow and fast twitch fibres are similar in the superficial and deep regions of human muscles On the other hand Johnson et al (1973) found that the fibre composition of deep and superficial parts of the lateral m gastrocnemius could differ considerably Determinations of fibre population in needle-biopsy specimens from approximately identical sites in the human triceps surae might seem valid However we found intraindividual differences in fibre composition (low and high ATPase activity respectively see Table 3) with right–left side differences in the soleus of up to eleven and in the lateral gastrocnemius head up to 40% Therefore we hold the view that for the triceps surae these intraindividual side differences which are eliminated in statistics are based on a heterogenous fibre distribution even though great care is taken to secure specimens from identical sites Consequently we dispute the validity of generalizations on compositional muscular homogeneity based on blindly obtained specimens However qualitatively for diagnoses of muscle pathology this technique has its given place

The wide variations in fibre population between these five healthy men of which none was actively engaged in competitive sports are in close agreement with the findings of other authors (Table 4) A general feature of both the soleus and the gastrocnemius muscles was the greater diameter of high— in relation to low—ATPase activity fibres Might this phenomenon in healthy males without special training reflect relatively high functional demands on this small population of fibres?

Structure and strength

Relationships between structure and strength in leg muscles have previously been demonstrated for knee-extensors in seated man where the one-joint m vastus lateralis has been subject to morphological analysis and the findings have been generalized as representative for the total knee extension force (cf Thorstensson et al 1976 1977) In analogy it might be expected that for the triceps surae and especially for the one joint m soleus which acts as a quadriceps surae (Campbell et al 1973) structure–strength relationships at least for static and slow-dynamic manoeuvres and specifically if the knee is fully extended could be demonstrated Such was however not the case and several factors must be discussed

Firstly none of our subjects were actively engaged in regular physical training and only in such individuals have relationships between functional capacity and structure been demonstrated (Saltin et al 1976 Thorstensson et al 1976 Bylund et al 1977 Thorstensson et al 1977) Secondly the traditional classification of muscle fibres based on stainability for ATPase is more an operational abstract than a real measure of fibre difference and not as such a reliable measure of the structural bases for the functional capacity (cf e g Edgerton et al 1975) Thirdly we do not with any degree of certainty know the relationship between fibre properties and the maximum angular force velocity Fr

strocnemius

l head		Lateral head	
Range/ S D		%	Range/ ± S D.
		63	56–69
48–57		50 deep	43–60
		44 superf	35–51
		53	38–73
		60	45–82
		57	
20		57	9
		68	14

ally we may in fact, never have reached sufficiently high speeds of angular motion that the possible specific properties of the high glycolytic fibres the triceps surae have dominated In analogy with other human organs such as li er kidneys lungs the triceps surae may also have great reserve capacity and we may especially if untrained use only part of it

This study was supported by grants from the Research Council of the Swedish Sports Federation, The Swedish Medical Research Council and the County of Västerbotten

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he effect of α-aminoisobuturic acid and 2 4-diaminobuturic cid on mouse blastocyst outgrowth in vitro

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NÆSLUND, G LINDQVIST I RONQUIST G. & NILSSON B O The effect of α-aminoisobuturic acid and 2,4-diaminobuturic acid on mouse blastocyst outgrowth in vitro. Acta Physiol Scand 1979 107 57–61 Received 15 March 1979 ISSN 0001-6772. Reproduction Research Unit and Department of Medical and Physiological Chemistry Biomedical Centre Uppsala, Sweden.

Blastocysts recovered from mice in state of delay of implantation were incubated for 10 h with either α-aminoisobuturic acid (AIB) or 2,4-diaminobuturic acid (DAB), two non-metabolizable amino acids. The incubation medium was composed so as to maintain growth arrest of the inactive delayed blastocysts in vitro The blastocysts were then transferred to complete outgrowth medium without the tw non-metabolizable amino acids to test the capacity for trophoblast outgrowth AIB which displays saturation kinetics, was harmles to the blastocysts even at high concentration, while DAB at low concentration irreversibly damaged the trophoblast cell and prevented outgrowth, probably due to nonsaturation kinetics resulting in high intracellular accumulation. The harmful effect of DAB could be abolished by concomitant incubation with L-alanine and L-methionine which compete with DAB for the same transport system, while the D-forms of the same amino acids had little or no effect The results suggest the presence of transport System A in mouse blastocysts growth arrested in vitro indicating an operative carrier mechanism already during delay of implantation.

Key words Mouse blastocysts in vitro α-aminoisobuturic acid, 2,4-diaminobuturic acid

In mice in a state of experimentally delayed implantation it has been found that after induction of implantation by an injection of oestrogen the in vivo uptake of the non-metabolizable ^{14}C-α-aminoisobuturic acid (^{14}C AIB) shows preference for the uterus (Lindqvist et al 1977) Further an in vivo uptake of ^{14}C AIB into the blastocysts themselves occurred 8–12 h after the induction of implantation provided that the blastocysts had been flushed out of the uterine cavity not earlier than 4 h after the ^{14}C AIB-injection (Lindqvist et al. 1978) Hence, there was a preferential uptake of ^{14}C AIB into uterine tissue during activation for implantation and a transport system for this non-metabolizable amino acid was apparently operating to transfer ^{14}C AIB from the uterine epithelium into the blastocysts via the uterine secretion

A similar transport system, the System A, is present in Ehrlich ascites tumour cells (Oxender & Christensen 1963) and also in cultured human glioma cells (Ronquist et al. 1976) This system is Na -dependent and energy-requiring. Short-chain neutral monoamino acids are the physiological substrates, but also diamino acids, such as the non-metabolizable 2,4-diaminobuturic acid (DAB) can share this transport system. However the diamino acids can usually be transported against a much steeper concentration gradient into the Ehrlich cells than the monoamino acids (Christensen et al. 1973) Therefore DAB contrary to AIB showed apparent non-saturation kinetics, resulting in damage to the Ehrlich cells (Christensen et al. 1952)

Delayed mouse blastocysts when flushed out of the uterine cavity and transferred to a serum-containing culture medium start to grow out, a process that has been considered to be analogous to implantation in utero (Gwatkin 1966*a*) whereas in a medium deprived of glucose, arginine and leucine

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Delayed mouse blastocysts when flushed out of the uterine cavity and transferred to a serum-containing culture medium start to grow out, a process that has been considered to be analogous t implantation in utero (Gwatkin 1966*a*) whereas in a medium deprived of glucose, arginine and leucine

the inactive delayed blastocysts are kept in a state of growth arrest and therefore remain expanded without signs of trophoblast outgrowth (Naeslund 1979)

The question arose whether delayed blastocysts when incubated in a medium that keeps them in a state of growth arrest would be damaged by the presence of AIB or DAB in the incubation medium indicating an operative carrier already before induction of implantation To test this mouse blastocysts from animals in delay of implantation were incubated with either AIB DAB or DAB together with two L amino acids that compete for the same transport system The blastocysts were then transferred to a serum containing outgrowth medium after 10 h and were observed by light microscopy to judge their viability and capacity for trophoblast outgrowth

MATERIAL AND METHODS

Pregnant mice of the NMRI strain (Anticimex Stockholm Sweden) were ovariectomized on day 3 (day 1 was the day a vaginal plug was found) and given 1 mg of the depot preparation medroxiprogesterone (Depo-Provera, Upjohn Co USA) subcutaneously to delay implantation (Yoshinaga & Adams 1966) Blastocysts were recovered not earlier than on day 7 when a steady state of blastocyst outgrowth activity is attained (Naeslund & Lundkvist 1978) The uterine horns were dissected free and the blastocysts were flushed into watch glasses with a syringe containing phosphate buffered saline with Mg and Ca according to Dulbecco (SBL, Stockholm Sweden) Dialysed foetal calf serum (Flow Laboratories, Irvine Scotland) was added in a concentration of 1% to prevent the blastocysts from becoming adhesive The serum was dialysed in a 100 times excess of a 0 15 M NaCl solution for 3 days with changes twice daily to remove the amino acids and glucose among other things, and was then filter sterilized.

Brinster's medium for ovum culture (Brinster 1963) was used but with 1 0 mg/ml of glucose instead of sodium lactate (Brinster & Thomson 1966) Reagent grade water from a Milli-Q System (Millipore Corp Bedford Mass. USA) was used This water had a specific resistance of about 18 MΩ/cm The medium contained 1 mg/ml of bovine albumin AIB DAB L-alanine L-methionine D-alanine and D-methionine were added in the combinations given in Table 1 Filter sterilized stock solutions containing 0 1 M of the amino acid were used with addition of 9 mg/ml of NaCl to render them isotonic To ascertain that the lack of essential amino acids did not damage the blastocysts a group of blastocysts was maintained in Brinster s medium with glucose but without all amino acids before being transferred to the outgrowth medium During incubation in droplets under liquid paraffin in an atmosphere of 5% CO_2 in air at 37°C (Brinster 1963) the blastocysts were examined in an inverted microscope to find out whether they were expanded to a normal extent or showed signs of damage. After 10 h they were transferred to an outgrowth medium namely Brinster's medium with glucose and with addition of the amino acids of Eagle s BME (Eagle 1955) and 1% dialysed foetal calf serum to ascertain the capacity for outgrowth of the trophoblast (Gwatkin 1966*b*) The blastocyst cultures were observed for 5 days by light microscopy Each series of experiments was repeated 2–6 times and comprised usually 16–28 blastocysts totally As a control blastocysts incubated with 10 mM DAB were always matched with blastocysts in other experimental groups

Table 1 *Effect of two non-metabolizable amino acids on blastocyst appearance and outgrowth*

The blastocysts were first maintained for 10 h in Brinster's medium for ovum culture containing the amino acids and were then transferred to an *outgrowth medium* N = blastocysts had a normal appearance and started to grow when transferred into the outgrowth medium, D = blastocysts damaged no ability for normal growth in the outgrowth medium

Amino acid concentration *in the incubation medium*	Number of blastocysts	Outcome at 10 h
10 mM AIB	18	N
25 mM AIB	20	N
1 mM DAB	17	D
10 mM DAB	78	D
10 mM DAB 5 mM L-alanine 5 mM L-methionine	20	N
10 mM DAB 5 mM D-alanine 5 mM D-methionine	16	D
Brinster s medium with glucose (lacking all amino acids)	6	N

RESULTS

The results are summarized in Table 1 AIB was harmless to the blastocysts regardless of its concentration within the range 10–25 mM while DAB at a concentration of 10 mM induced a gradual collapse of the blastocysts starting after 3–4 h followed by lysis of the trophoblast cells (Fig 1*a*) These cells therefore displayed no ability to grow out after being transferred to the outgrowth medium when examined daily for 5 days The blastocysts were damaged in a similar way in 1 mM DAB but not so severely as a few trophoblast cells grew out after transfer to the outgrowth medium The outgrowths were however quite small and abnormal

Concomitant incubation with 5 mM L-alanine and



Fig. 1. ( ) A blastocyst incubated in 10 mM DAB. The blastocyst is disintegrated. (*b*) A blastocyst incubated in 10 mM DAB in the presence of L-alanine and L-methionine. The blastocyst is expanded. ( ) Two blastocyst incubated as the blastocyst in Fig. 1*b* 2 days after transfer to the outgrowth medium. Trophoblast cells are growing. The bars indicate 50 µm. Bright field (and *b*) and phase contrast ( ) microscopy.

mM L-methionine prevented the effect of 10 mM DAB. Hence, the normal-looking expanded blastocysts showed normal outgrowths in the outgrowth medium after 2 to 3 days (Figs. 1*b* and). The D-analogues of methionine and alanine also prevented the DAB effect but t a much lower degree. Four out of 16 blastocysts were expanded and the expanded blastocysts only displayed small outgrowths.

Blastocysts that were maintained in Brinster's medium with glucose and albumin but lacking all amino acids were expanded and had a normal appearance after 10 h and showed normal outgrowth after transfer to the outgrowth medium.

DISCUSSION

The effect of AIB and DAB on the blastocysts was evaluated in the present experiments from the capacity of the trophoblast cells of delayed blastocysts to grow out in a serum-containing complete medium after incubation with the respective amino acids. Since the response by the trophoblast cells under the prevailing conditions was unequivocal no graded scaling was decided necessary.

In attempts to achieve as standardized conditions for outgrowth as possible blastocysts flushed out of the uteri from mice in an experimental state of delayed implantation were used. The use of delayed mouse blastocysts in tests of trophoblast outgrowth

offers methodological advantages These blastocysts no longer possess a zona pellucida (McLaren 1970) which excludes interference of hatching with the outgrowth Further these blastocysts have a defined capacity for outgrowth (Naeslund & Lundkvist 1978)

The observed blastocyst destruction is most probably due to an extensive accumulation of DAB This accumulation will lead to exertion of increased osmotic pressure inside the cell by the non-metabolizable amino acid This high osmotic pressure and perhaps the lowered adenylate charge potential (Atkinson 1977) both caused by the energy-dependent accumulation of DAB will eventually result in lysis of the blastocysts An accumulation of an artificial amino acid inside the blastocysts does not necessarily per se lead to destruction or inhibited outgrowth of the blastocyst As observed AIB in the medium did not impede subsequent growth after transfer of the blastocysts into the outgrowth medium This is explained by the saturation kinetics displayed by AIB which result in a lower intracellular accumulation of this amino acid as compared with DAB

The transport system involved is most probably the Na -dependent energy-requiring System A since the DAB effect can be abolished by L alanine and L methionine which are both to a large extent transported by this system (Oxender & Christensen 1963) The present effect is most probably explained by competitive inhibition between the model amino acid and the two physiological amino acids for the same site at the receptor carrier complex Accordingly the D-analogues of alanine and methionine which have a lower affinity for the carrier (Oxender & Christensen 1963) interfered with the DAB transport to a much lower extent

To prevent activation in vitro of the inactive delayed blastocysts it would seem suitable to use a medium containing no glucose arginine or leucine since such a medium keeps the delayed blastocysts in a state of growth arrest (Naeslund 1979) In the present experiments the medium had to contain glucose since an energy requiring transport mechanism was beeing studied during a relatively long observation period but the absence of all essential amino acids however probably prevented the glucose from causing activation of the blastocysts Further the effect of DAB was observed after only a few hours in culture indicating that the carrier mechanism is already operative in the delayed blastocysts This is reasonable since delayed uterine blastocysts are known both to increase their weight (Hensleigh & Weitlauf 1974) and to possess many endocytotic vesicles (Schlafke & Enders 1973) both features indicating metabolic activity

Further in vitro studies of the transport characteristics for ^{14}C AIB on blastocysts recovered during delay and activation for implantation would reveal whether the increased uptake after induction of implantation (Lindqvist et al 1978) is caused by a new synthesis of carriers in the plasma membrane or by a changed affinity of the receptor-carrier complex for the amino acids Such studies are under way

This study was supported by grants from the Swedish Medical Research Council project No 1-X 70 and 14 B76-13X 228-1 A We thank Mrs Barbro Einarsson for skilful technical assistance Depo-Provera was kindly supplied by Upjohn AB Sweden

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ᑭartition of ^{125}I-iodoantipyrine among erythrocytes ɔlasma and renal cortex in the dog

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CLAUSEN G. HOPE, A & AUKLAND K., Partition of ^{125}I-iodoantipyrine among erythrocytes, plasma, and renal cortex in the dog. Acta Physiol Scand 1979 107 63–68 Received 17 March 1979 ISSN 0001-6772. Institute of Physiology University of Bergen, Norway

The tissue/blood partition coefficient, λ_{tb}, defined as the amount of blood having the same tracer content as one unit of tissue at diffusion equilibrium was determined for ^{125}I-iodoantipyrine (I Ap) and tritiated water (THO) in the dog kidney cortex. Measurements were made after in equilibration for 75 to 300 s and with liver circulation excluded. In 18 kidneys, λ_{tb} for I Ap averaged 1.38 (S.D. 0.11) w/w (weight/weight), without significant correlation to hematocrit (range 23–43) or to urine pH (range 5 5–8 6). The λ_{tb} for THO averaged 0 97 (S.D 0 06) v/w (volume/weight), close to the relative water contents. Erythrocyte/plasma partition for I-Ap was 0 82 w/w compared to water partition of 0.72. Thus, at diffusion equilibrium the apparent I-Ap concentration in renal cortical and red cell water exceeds that of plasma water by 14 and 60 % respectively It follows that I-Ap cannot be used as general indicator for total tissue water content. When used for measurement of local blood flow ad modum Kety λ_{tb} must be determined for each tissue and species.

Key ords Distribution volume tissue water renal blood flow renal medulla, tritiated water

)ue to its lipid solubility and rapid distribution ntipyrine has been used to estimate total water ontent of the whole organism or of individual rgans based on the assumption that it distributes venly and exclusively in the whole intra- and extracellular water phase A similar distribution has been eported for ^{125}I-iodoantipyrine (I-Ap) and both ubstances have been used for measurement of ocal blood flow with the so-called tissue sampling echnique ad modum Kety (1960) In accordance erewith, recent in vivo measurements of the issue/blood partition coefficient (λ_{tb}) for the rat idney gav value close to 1 00 and plausible alues for local renal blood flow (Hope Clausen Aukland 1976). However preliminary flow measurements in the dog kidney using the same partition coefficient gave absurdly high cortical flow ates, prompting the present examination of the Ap distribution space in the uog ..dney

While I-Ap is readily available nonvolatile and easily quantitated it has other less desirable qualities which have to be taken into account: Several investigators have reported a rapid loss of the iodine tag in vivo as well as metabolic conversion of the antipyrine molecule itself (Soberman et al 1949 Straub et al. 1964) The liver has been blamed for both processes Furthermore slowly saturating intracellular compartments for antipyrine have been suspected (Hilton & Dallam 1969 Effros & Chinard 1969)

For these reasons and in order to mimick the conditions during flow measurements (8 to 20 s infusion) we decided to measure the kidney tissue/blood partition coefficient (λ_{tb}) (1) in vivo (2) without circulation through the liver and (3) with the shortest saturation period compatible with complete diffusion equilibration between arterial blood and tissue.

For the purpose of methodological control λ_{th} for THO (tritiated water) was determined by the same experimental procedure

METHODS

Preparation and control of [125]*I-iodoantipyrine*

[125]I-Iodoantipyrine (I Ap) in 0 2% NaHCO solution was obtained from New England Nuclear Chicago The amount of free [125]I was determined according to New England Nuclear specifications by silica gel thin layer chromatography (Polygram SIL N HR Machery Nagel) with chloroform–ethyl ether (85 15) as eluent Since free [125]I often exceeded the amount recorded by the manufacturer shortly before shipping (1–5%) the preparation was purified on a Sephadex G-10 column with diameter 0 9 cm and height 18 cm using distilled water for elution (Hope et al 1976, Munck & Andersen 1967) At the time of use the free iodine content was usually 1–2% and never exceeded 5% Final dilution in saline to about 5 μCi/ml was made shortly before infusion

Experimental procedures

Experiments were performed on 13 mongrel dogs weighing from 15 to 23 kg. Anesthesia was induced by i.v Nembutal 25 mg/kg b wt and supplemented with doses of 3–5 mg/kg when needed An endotracheal tube was inserted but the ventilation was not assisted After anesthesia the dogs were given a constant i v infusion of 0 9% NaCl and 3% mannitol at a rate of 4 ml/min Para-aminohippurate (PAH) was added to provide an arterial concentration of 10–20 mg/l In three experiments an additional infusion of 5% NaHCO was given to obtain alkaline urine and in one experiment dilute HCl was infused for urine acidification The abdomen was opened by a midline incision and polyvinyl catheters were inserted in both ureters A nylon snare was placed loosely around each renal pedicle with as little dissection as possible The spleen was removed the portal vein dissected free and nylon snares were placed loosely around the origin of the celiac and superior mesenteric arteries Under short lasting occlusion of these arteries, a shunt was established from the portal vein to the left common iliac vein by means of polyvinyl tubing, diameter 8 mm. Pressure in the shunt was measured through a T-connection to ascertain free drainage of splanchnic blood Arterial pressure was measured with a Hewlett Packard transducer through a catheter in a brachial artery A PE 120 tube was inserted into the femoral artery for blood sampling. After establishing the porta-iliac shunt PAH clearance was determined on each kidney in two or three consecutive 10 min periods, arterial blood samples being drawn at the midpoint of each period In order to stop also hepatic arterial blood flow the snare on the coeliac artery was tightened A couple of minutes later an i v infusion of I Ap was started. The infusion rate was gradually reduced after a predetermined schedule such as to maintain arterial concentration at a constant level Simultaneously arterial blood samples were collected continuously at 5 s intervals by free drainage from the femoral artery catheter After 75 to 300 s infusion the renal pedicles were clamped

The kidneys were rapidly removed and frozen in CO ethanol and thereafter stored at −70°C

The procedure described above was followed in 9 ex with analyses on a total of 13 kidneys. In 4 expts (5 ki neys analysed) the procedure was simplified by omi the portailiac shunt Instead, liver blood flow was stop by clamping the superior mesenteric and the coe arteries as well as the portal vein a couple of min before tracer infusion Hemodynamic reactions to t procedure such as changes in arterial pressure and in renal blood flow (Nycotron electromagnetic flowme were found to be small and transitory

The λ_{th} for THO (tritiated water) was determined i of the expts described above and in 3 additional ex without interference with liver blood flow

Treatment of tissue and blood samples

Tissue samples were obtained from the still frozen kid at 4°C as follows 5–7 mm thick saggital sections were c by saw and thereafter 12 to 40 samples of 0.1 to 0.3 were dissected out by scalpel from the middle two thi of the renal cortex Each sample was immediately tra ferred to a preweighed counting vial weighed again, a added 4 ml distilled water Equilibration of I Ap and TH was found to be complete after 24 h After determi I Ap activity on the whole sample 100 µl of each sam was transferred to vials containing 6 ml scintillation flu for measuring THO activity

Blood samples of 0 1 to 0 3 ml for measurement of I activity were pipetted into preweighed counting vi weighed again and added 4 ml distilled water To a cumbersome bleaching procedures plasma instead whole blood was used for determination of THO act One glass capillary was filled from each blood sample spun in a hematocrit centrifuge About 0.02 g plasma transferred to preweighed counting vials which were weighed again and added 6 ml scintillation fluid Calc tion of whole blood activity was made on the assump of uniform activity in plasma and red cell water a plasma and whole blood water content determined in e experiment as described below

Calculation of λ_{ta}

Since it is not possible in every experiment to ensure co plete equilibration between tissue and the last bl sample λ_{ta} was calculated by a method which takes i account the whole arterial concentration curve as wel the actual renal blood flow (Hope et al 1976); Cort blood flow was roughly estimated from the PAH clear (C_{PAH}) under the assumption of 75% extraction a cort flow fraction of 0 90 and cortical volume=0.7 × kid weight (KW)

$$F/W = \frac{C_{PAH}\ 0.9}{(1-\mathrm{Hct})\ 0.75\ KW\ 0.7} \qquad (4)$$

PAH in plasma and urine was determined by the meth of Smith et al (1945)

Under the assumption of complete equilibration b tween tissue and venous blood the tissue concentration an inert tracer (C) at time T is given by the follow equation (Kety 1960)

$$u(T) = \lambda_{ab} k_i \int C_a e^{-k_i(T-t)} dt \qquad \text{(eq. 2)}$$

here C_a is tracer concentration in arterial blood. k_i ... tissue saturation rate constant, equals $F/(W \cdot \lambda_{ab})$, here F/W is blood flow per unit weight, in casu cortical ... flow obtained from PAH clearance (eq. 1). By ...oosing various values for λ_{ab}, and using the k_i resulting ... F/W and the chosen λ_{ab}, corresponding values for ...(T) were obtained from eq. 2. Thereafter the λ_{ab} fitting ...e observed $C_i(T)$ was derived by interpolation (Hope ... al. 1976).

...rythrocyte/plasma ...ater and I-Ap partition

...lood from 8 dogs given I-Ap for flow measurements ... separated by centrifugation in 10 ml tubes for 10 min at an average g of 1800. ¹²⁵I radioactivity as well as water content (weighing and drying for 48 h at 90°C) was determined in whole blood, plasma, and packed red cells. ...ematocrit was determined by standard microtube centrifugation.

...ipid solubility of I-Ap

...he olive oil/water partition of I-Ap was examined by ...ixing and shaking equal volumes of water containing ...Ap (0.5% free ¹²⁵I) and olive oil at room temperature. ...fter three minutes equilibration, water and oil were separated by centrifugation and ¹²⁵I activity was measured in ...e two phases.

...otope measurements

...-Ap activity was determined in a well scintillation gamma ...ounter and THO in ... Mark I or Isocap 300 liquid scintillation system (Nuclear Chicago) using external standard ...o correct for quenching. Interference by ¹²⁵I liquid scintillation was minimal because of ... 10³ times higher activity ...f THO in the infusate.

...fedullary blood flow

...etermination of λ_{ab} for medullary tissue would require ...uch longer tracer infusion, and was therefore not made. However the present infusion time is ideal for estimation ...f inner medullary blood flow and samples from inner ...edulla were therefore dissected in most experiments. ...ocal flow in ml/min per g tissue was calculated from ...q. 2.

RESULTS

...ince I-Ap was determined per gram blood and ...issue $\lambda_{ab\ I-Ap}$ has the dimension of w/w (weight/weight). THO activity was determined per gram ...ssue and per ml blood, giving $\lambda_{ab\ THO}$ the dimension ...f v/w (volume/weight).

Tissue/blood partition of I-Ap

The partition coefficient for each kidney was calculated from the average I-Ap concentration of 12 ...to 40 cortical tissue samples. The concentration ...scatter in individual kidneys was small, with coefficients of variation ranging from 1.4 to 5.6% (Avg. 3.2%) attesting to uniformity of equilibration and partition coefficient within the cortex.

Two experiments with infusion times of 75 and 300 s respectively shown in Fig. 1 demonstrate clearly a much higher I-Ap activity in the renal cortex than in the last arterial blood samples, indicating a tissue/blood partition coefficient well above unity. The average calculated for 18 kidneys in 13 dogs was 1.38 (S.E. of the mean 0.03). The range was 1.17 to 1.61 and the S.D. 0.13. The individual average for the 13 dogs (2 kidneys in 5 dogs) was 1.35 (S.D. 0.12).

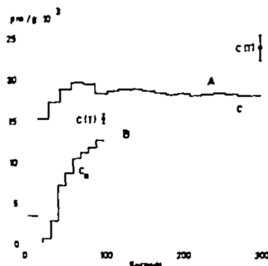


Fig. 1. Two arterial concentration curves (C_a) and corresponding tissue concentrations ($C_i(T)$, mean ± 1 S.D.) for ¹²⁵I-iodoantipyrine. Calculated λ_{ab}: 1.31 in expt. A, 1.39 in expt. B.

As evident from Fig. 2, λ_{ab} tended to increase with increasing hematocrit, but the correlation was not statistically significant ($p > 0.05$). The λ_{ab} obtained in 3 expts. with saturation periods of 300 s (1.29, 1.35 and 1.39) did not deviate from that of the remaining 10 experiments with 75 to 130 s infusion. No relationship to cortical blood flow, to the shape of the blood concentration curve, or to uniformity of cortical sample concentrations from each kidney could be detected. Furthermore, as evident from Fig. 2, $\lambda_{ab\ I-Ap}$ showed no correlation to urine pH in the range of 5.5 to 8.6.

Erythrocyte/plasma partition of water and I-Ap

The unexpectedly high λ_{ab} for I-Ap might be due to a lower I-Ap concentration in red cell water than in

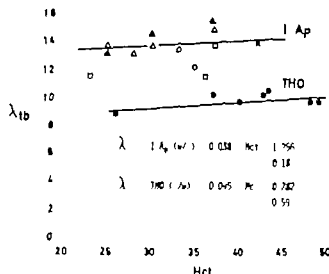


Fig 2 In vivo partition coefficients for THO (closed circles) and for I Ap as related to hematocrit The various symbols for I Ap indicate urine pH Open circles, pH<6.5 Triangles, 6 5<pH<7 5 Squares, pH>7 5 Crosses pH unknown

plasma water λ_{cp} (red cell/plasma partition) for I Ap and water was therefore determined in blood from 8 dogs infused with I Ap The observed λ_{cp} for I Ap (w/w) averaged 0 85 (S D 0 05) or 0 8? when corrected for trapped plasma Hematocrit averaged 42 (range 3? to 47) The water content (w/w) averaged 0 9?7 (S D 0 006) in plasma 0 678 (S D 0 017) in packed red cells (10 ml tubes) and 0 81? (S D 0 019) in whole blood Taking the corresponding specific weights to be 1 025 1 090 and 1 050 gives an average red cell water content of 0 657 indicating 4% trapped plasma in packed cells and a true λ_{cp} for H_2O of 0 7? (w/w) The I Ap/H_2O partition ratio 0 82/0 7?=1 14 thus indicates that the apparent I Ap concentration in red cell water is 14% higher than that in plasma water Similarily it may be calculated that the apparent I Ap concentration in kidney tissue water is 60% higher than that of plasma water at diffusion equilibrium

Lipid solubility of I Ap

The olive oil/water partition coefficient of I Ap activity at room temperature averaged 1 08 v/v (S D 0 06 $n=9$) Correction for free iodine (assumed to be insoluble in oil) gave a true I Ap partition of 1 16

Tissue/blood partition of THO

The variation of THO concentration among tissue samples from the same kidney was about twice that for I Ap with an average coefficient of variation of 7 1% (range 3 0 to 12.6 in 8 kidneys) The average tissue/plasma partition coefficient (λ_{tp}) was 1? (S D 0 05) v/w Conversion to tissue/blood partition (λ_{tb}) was made on the assumption of a red cell/plasma THO distribution equal to that of water, plasma water content of 0 95 (v/v) and individual hematocrits (Avg 41 range 26 to 49) and tissue water contents (Avg 0 854 S D 0 018 v/v) The resulting λ_{tb} averaged 0 97 (S D 0 06) v/w As shown in Fig 2 λ_{tb} for THO tended to increase with increasing Hct but the correlation was not statistically significant ($p>0$ 05)

Inner medullary blood flow

The infusion periods of 75 to 300 s resulted in I to 60% saturation of the inner medulla Using the observed cortical partition coefficients average inner medullary "blood flow" estimated from I Ap and THO uptake in 15 kidneys was 0 74 (S D 0 15) ml/min g No difference between I Ap and THO was observed in 6 expts in which both tracers were determined Cortical blood flow estimated from PAH clearance (eq 1) averaged 4 39 with range of ? 10 to 6 1 ml/min g

DISCUSSION

The average I Ap partition coefficients of 1.3? for kidney/blood and 0 85 for red cell/plasma, contrast with the common view that the distribution space for antipyrine and I Ap in cells and tissues is equal to that of water (Hope et al 1976 Krasnow et al 1963 Talso et al 1955) or even lower (Brightman et al 1971) However some literature data suggest differences both among species and among tissues and the present findings are not without precedence For dog erythrocytes Effros & Chinard (1969) found a 10% higher apparent I Ap concentration in cell water than in plasma water in good agreement with the present figure of 14% while Carlin & Chien (1977) recently reported a surplus of 40% in red cell water We have no explanation for this discrepancy In any case the high solubility of I Ap in erythrocytes with a cell/plasma partition close to unity should implay a tissue/blood partition coefficient rather insensitive to hematocrit changes in agreement with the in vivo experimental findings (Fig 2)

For the dog kidney Soberman et al (1949) found

10% higher concentration of antipyrine in tissue water (two kidneys examined) than in plasma water while Huckabee (1956) reported a surplus of 19% (one kidney examined) for aminopyrine. Appreciably higher values were obtained in goat and man. Obviously a direct comparison with I-Ap is questionable since the observed fat solubility of I-Ap is at least 15 times higher than that reported for antipyrine (Renkin 1957). No previous measurements seem available for I-Ap partition between kidney tissue and blood in the dog. However our previous finding of $\lambda_m = 1.00$ for the rat (Hope et al. 1976) and the present rather great individual scatter around the surprisingly high average of 1.38 call for close scrutiny of the methods.

Errors due to free iodine or other breakdown products of I-Ap would seem to have been reduced to a minimum by (1) excluding the hepatic circulation, and (2) by using the shortest possible infusion period compatible with practically complete equilibration between arterial blood and the renal cortex (5 saturation half times or more). Extending the infusion period from 75 to 300 s had no consistent effect on the estimated λ_m, giving no evidence for slowly saturating intracellular compartments within this period of time.

The method used for calculating λ_m may need some comments. As a first approximation λ_m was estimated as the ratio between the observed tissue concentration and the average concentration of the last three blood samples. However because of the infusion time and somewhat changing arterial concentration, a representative arterial value might not be obtained in this way. An intuitive correction for the lack of complete equilibration could be made based on the actual arterial curve and knowledge of the approximate saturation rate constant. Thus, in the case of increasing final arterial concentration one would choose a somewhat lower arterial concentration for comparison with tissue than in the case of falling arterial concentration. The method described above for calculating λ_m replaces this subjective correction by including the whole arterial concentration curve as well as cortical saturation rate derived from PAH clearance. It should be emphasized that the accuracy of the cortical flow estimated from eq. 1 is not critical to the calculation of λ_m. Thus, in both experiments shown in Fig. 1 an error in cortical flow of 20% would change the estimated λ_m by less than 0.01. (The intuitive approach outlined above would usually deviate by less than 0.05 from the calculated value.)

The fact that the pH of certain compartments of the kidney may deviate greatly from that of other biological fluids made us suspect some association between I-Ap uptake and tubular fluid acidification, but experimental evidence did not support that suspicion (Fig. 2).

The finding of a λ_m for THO of 0.97, close to that expected from the average water contents of cortical tissue and blood, attests strongly to the validity of the methods and of the measured λ_m for I-Ap. However in spite of smaller analytical errors for I-Ap than for THO in tissue and blood (cf. coefficients of variation for individual cortical samples), λ_m for I-Ap showed greater scatter than that for THO, suggesting individual differences in the renal I-Ap space.

In conclusion. Having excluded gross methodological errors, we must accept that I-Ap has a much higher solubility in kidney tissue than in blood. In fact, calculations show that in comparison to plasma, the tissue has an apparent distribution volume about 60% greater than its water content. The finding is especially surprising in view of the λ_m close to unity measured with the same technique in the rat kidney (Hope et al. 1976), but we have no plausible explanation for this species difference.

Knowledge of λ_m is necessary for estimating local blood flow according to Kety (1960). Since the error in calculated flow resulting from an inappropriate λ_m will increase with increasing degree of saturation (Eklöf et al. 1974, Hope et al. 1976), the apparent interindividual variations in λ_m for I-Ap suggest the use of short infusion periods. On the other hand the effect on calculated flow of inaccurate timing of the infusion duration (about 10 s) will be greater with short infusion periods (Clausen et al. 1979). However the higher λ_m for I-Ap than for THO implies that the infusion period for I-Ap may be prolonged by 40% compared to that of THO to obtain a given degree of saturation, thus adding to the analytical advantage of I-Ap over THO.

The values for inner medullary "blood flow" obtained as a byproduct of these experiments should be considered with reservations. (1) The uptake rate is undoubtedly influenced by counter current exchange and urine flow and does therefore reflect "nutritive flow" rather than "true blood flow". (For further discussion see Aukland & Berliner 1964, Hope et al. 1976, Clausen et al. 1979.) (2)

Total renal blood flow was somewhat lower than we usually observe under control conditions probably because of the rather extensive surgery and the porta-iliac shunt

While the present study is primarily of interest for the use of I Ap for measuring local renal blood flow in the dog it also permits some general con clusions The distribution space for I Ap may deviate greatly from that of water in a given tissue Furthermore this deviation may vary considerably among tissues and even among species

Added in proof In agreement with the present data Sakurada et al. (Amer J Physiol 234 H59-H66 1978) re ported a much higher solubility for iodo[^{14}C]antipyrine than for [^{14}C]antipyrine in nonpolar solvents However the brain/blood partition coefficients for both substances 0 80 and 0 91 respectively differ markedly from our results on the renal cortex

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·istribution of blood flow in the dog kidney

Saturation rates for inert diffusible tracers, ¹²⁵I-iodoantipyrine and tiated water versus uptake of microspheres under control conditions

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CLAUSEN G HOPE, A KIRKEBØ A., TYSSEBOTN I. & AUKLAND, K. Distribution of blood flow in the dog kidney I Saturation rates for inert diffusible tracers, ¹²⁵I-iodoantipyrine and tritiated water versus uptake of microspheres under control conditions. Acta Physiol Scand 1979 107 69–81 Received 17 March 1979 ISSN 0001-6772. Institute of Physiology University of Bergen, Norway

Disparate reports on intrarenal blood flow distribution prompted direct comparison between microspheres (Ms) and inert diffusible tracers (DT). The "tissue sampling technique for estimating local flow with DT (Kety) was adapted for the dog kidney using ¹²⁵I-iodoantipyrine (I Ap) and tritiated water (THO). M (15 μm) were injected 3 min prior to 10–15 DT infusion made during continuous 1 arterial blood sampling. Tracers were measured in 7 to 20 samples from each of the following zones: Outer middle and inner cortex (C_1 C_2, C_3) outer and inner halves of outer medulla (OM OM), and inner medulla (IM). I-Ap and THO gave closely similar flow distributions, and average total renal blood flow (RBF) of 3 90 and 3 78 as compared to 3 94 ml/min g with Ms. Flow in C_1 (ml/min g) was similar with all tracers, and in per cent thereof verage local flows were: C_2 102, C_3 70, OM 34 OM_2 12, and IM 2 with DT versus 117 53, L 3 and 0 with Ms. Zonal flow fractions of total RBF obtained with DT were: C_1 0.41 C_2 0.33 and C_3+medulla 0.26 versus 0 51 0.33 and 0.16 with Ms. Thus, Ms surplus in C_1 relative to DT flow representing 10% of total RBF matched a Ms deficit in C_3 medulla. This disparity might result from: (1) Failure of M to enter deep afferent arterioles in proportion to blood flow (2) diffusion of DT from deep portions of the interlobular arteries, and/or (3) postglomerular low ard flow of blood and DT

Key ords. Renal blood flow kidney circulation, local flow measurement, inert diffusible tracers, microspheres

It is commonly assumed that changes of the intrarenal distribution of blood flow may influence several renal functions However the verification of such hypotheses is hampered by the fact that various methods for measuring local or regional blood flow in the kidney give different results. This clearly calls for scrutiny of the flow techniques, and two principally different methods will be explored here: (1) The rate of uptake (or removal) of inert diffusible tracers (Kety 1951) and (2) the distribution of intra-arterially injected microspheres (McNay & Abe 1970)

(1) In the case of diffusible tracers the inert gases H_2 and ⁸⁵Kr (or ¹³³Xe) offer the great advantage of practically unlimited repeatability of measurements (Aukland Bower & Berliner 1964 Thorburn et al 1963) However with both methods objections may be raised against the techniques used for recording local gas concentration. The H method involves the trauma of inserting platinum electrodes into the tissue. The local trauma is avoided with external monitoring of ⁸⁵Kr-washout, but the deduction of local concentration patterns from the composite desaturation curve involves great uncertainties. For these or other reasons the results obtained with the two methods differ considerably both under control conditions and in various experimental conditions, as recently reviewed by one of us (Aukland 1976).

(2) While the use of microsphere uptake has been

widely accepted It should be realized that there is only indirect evidence that the microspheres distribute in proportion to blood flow within the kidney It is especially conspicuous that renal vasodilation invariably causes an inproportionate rise in microsphere uptake in deep cortex while no such redistribution has been observed with diffusible tracers (For references see Aukland 1976)

Because of these discrepancies we decided to study the local uptake of inert diffusible tracers in the dog kidney by the "tissue sampling technique" (Kety 1951) providing atraumatic flow measurements in *well defined anatomical regions* This approach permitted *simultaneous measurements of microsphere uptake* in exactly the same locations

Since the volatility of gases may cause considerable technical problems we decided to use [125]I iodoantipyrine (I Ap) and tritiated water (THO) The uptake of these tracers is commonly assumed to be exclusively flow limited and in the case of I Ap we have recently obtained evidence that this holds true for the rat kidney (Hope Clausen & Aukland 1976 Clausen & Hope 1977)

A brief account of some of these experiments has *appeared in abstract form (Clausen et al 1977)*

METHODS

Inert diffusible tracers Tissue sampling technique

Principle The basic assumption underlying the method is that highly diffusible tracers entering the tissue through *arterial blood will reach complete equilibrium with the* tissue in one single passage In other words that the tracer activity is equal in venous blood and in the tissue *it is drained from As shown by Kety (1951) blood flow* per unit tissue weight (*F/W*) may be estimated from the *tracer* concentration in the tissue (C_i) at time T arte*rial tracer concentration (C_a) in the period 0 to T and the* tissue/blood partition coefficient (λ_m)

$$C_i(T)=\lambda_m k\int_0^T C_a e^{-k(T-t)}\,dt \qquad (1)$$

where the rate constant $k=F/(W\lambda_m)$ Thus if λ_m is known and $C_i(T)$ and the C_a-curve from time zero to T is determined tissue blood flow can be estimated Since eq (1) cannot be solved mathematically a curve is constructed for corresponding values of $C_i(T)$ and k_i using the arterial concentration curve for each particular experiment (Kety 1960)

Tissue/blood partition coefficients The tissue/blood partition (λ_m) for I-Ap i e the amount of blood having the same I Ap content as one gram of tissue provided complete diffusion equilibrium has been shown in a separate study to be higher in the dog kidney than in any other tissue studied so far (Clausen et al 1979) In vivo equilibration for 75 to 300 s gave an average λ_m of [illegible] (S D 0 13 $n=18$) w/w (weight/weight) for the renal cortex without statistically significant correlation to hematocrit *Since I Ap activity was determined per gram blood and tissue* and λ_m has the dimension of w/w eq (1) will give flow in gram per min per gram tissue Conversion *to ml/min g was made by dividing by the specific gravity* of blood 1 05

Tissue blood partition for THO determined in the same in vivo expts averaged 0 97 (S D 0 06) v/w (volume/weight) For practical reasons a value of 1 00 was used

Preparation of [125]I-iodoantipyrine [125]I-iodoantipyrine (I Ap) in 0.[illegible] % $NaHCO_3$ solution was obtained from New England Nuclear Chicago Free iodine was removed on a Sephadex G-10 column and the purity tested by silica gel chromatography as described elsewhere (Hope et al 1976 Clausen et al 1979) At the time of use the free [125]I content was usually 1 [illegible] % and did not exceed [illegible] Final dilution in saline to an activity of about 5 µCi/ml was made shortly before injection of a 50 ml dose.

Experimental protocol All experiments were performed on mongrel dogs (b wt 15 to 25 kg), fasted over night but with free access to water Anesthesia was induced by i v injection of Nembutal 25 mg/kg b w and supplemented with doses of 3 to 5 mg/kg when needed An endotracheal tube was inserted to secure free air ways Respiration was not assisted Polyethylene catheters were inserted into a femoral vein for infusion and in an artery for pressure recording with Hewlett Packard transducer and recorder

Two series of blood flow measurements will be reported *Series A* includes 9 expts with I Ap as the only *tracer One kidney was exposed retroperitoneally through* a flank incision The ureter was catheterized and a nylon sling placed loosely around the renal pedicle I-Ap was *given as a constant i v infusion over a period of* [illegible] 20 s One s arterial blood samples were collected from a femoral artery PE 60 catheter as described below The *catheter length was 30 cm,* and the tip was located about 15 cm from the entry in the femoral artery i e approximately at the aortic bifurcation. Catheter volume was [illegible] *ml*

Series B includes 10 experiments with practically simultaneous injection of I Ap/THO and microspheres (Ms) *Both kidneys were exposed retroperitoneally* and nylon snares were placed loosely around the renal pedicles. One renal artery was provided with an electromagnetic flow *probe (Nycotron Oslo) and catheterized* with a thin polyvinyl tube for infusion of acetylcholine A catheter was placed in the left ventricle through the right carotid *artery for tracer injections About 5 min* before beginning the experiment to be reported here one Ms batch had been injected under control conditions Thereafter under continuous *intra-arterial infusion of Ach* in one kidney a second Ms batch was injected into the left ventricle, followed within 3 min by a constant infusion of I Ap/THO into the same catheter The doses of I Ap and THO were 50 µCi and 50 *mCi* respectively Arterial blood was sampled from a 50 cm long femoral catheter the tip being placed in the aorta approximately at the level of the renal arteries Both renal pedicles were clamped simultaneously after 10–15 sec infusion.

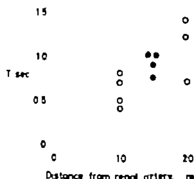


Fig. 1. Difference in appearance times (ΔT) of cold saline in the aorta at the origin of the renal arteries and 10, 15 and 20 cm further downstream.

This report will give data for blood flow distribution in the control kidney and total flow in both kidneys as estimated from the three tracers. The effect of Ach on flow distribution will be described in a following paper.

Position of arterial sampling catheter. Unexpectedly high cortical flow in a couple of experiments in series A (short sampling catheter) made us suspect that the recorded arterial concentration curve might be more delayed compared to that delivered to the kidney than could be accounted for by catheter passage alone. The transit time in the abdominal aorta was therefore tested in two experiments as follows: Two thermocouples, each mounted at the tip of a heart catheter, were introduced into one femoral artery. The temperatures were recorded continuously on a Rikadenki recorder using a thermocouple in ice water as reference. One probe was advanced 30 cm from the insertion on the thigh, approximately to the level of the renal arteries while the second was placed alternately 10, 15 and 20 cm further downstream. Slug injection of 3–5 ml saline caused a sharp deflection at each probe, and the time lag was recorded for the first appearance (calculation from the main temperature downstroke retropolated to baseline gave identical results). As shown in Fig. 1, sampling at the aortic bifurcation or in the iliac artery may cause a delay of more than 1 s. We have not found it permissible to introduce a correction for this delay but calculations showed that flow may have been overestimated by up to 15%. In series B we therefore used a 50 cm long sampling catheter (PP 120, volume 0.40 ml), the tip being placed 30 cm from the entrance at the thigh. We thereby obtained a measurable delay relative to renal arterial blood and could make appropriate correction of the arterial concentration curve. A usual sampling flow rate would be 0.4 ml/s, giving a mean catheter transit time of 1 s.

"Catheter smearing" was tested as described previously (Hope et al. 1976). Correction of the arterial concentration curve had little influence (magnitude 1%) on calculated flow and was therefore omitted.

Blood and tissue sampling. Blood sampling was started simultaneously with I-Ap/THO infusion by cutting the aortic catheter 15 to 20 cm from its exit from the femoral artery. One sec samples were collected manually into a row of preweighed heparinized vials, and continued until 2–3 s after clamping the renal pedicles. Catheter flow rate and mean transit time were calculated from the timed sample weights and catheter volume. While still clamped, the kidneys were rapidly excised, weighed, plunged into CO_2-ethanol, and were thereafter stored at −70°C.

Tissue samples were dissected out at 4°C as follows: 5–7 mm thick transversal or sagittal slices were cut out by circular saw. After scraping off "saw dust" with a scalpel, 7–10 mm broad sectors perpendicular to the kidney surface were cut out and thereafter divided into appropriate zones. The arcuate arteries were used as landmarks for the cortico-medullary border. As a further check, the section was also inspected under low-power magnification for vasa recta bundles as indicators for outer medulla. The cortex was usually divided in three zones (C_1, C_2 and C_3 from capsule to corticomedullary border), and in some experiments of series A in two zones (OC and IC, outer and inner cortex) of equal thickness (Fig. 2). The border to the inner medulla was judged from the color change and loss of striation. Usually outer medulla (OM) was divided in two layers of equal thickness (OM_1 and OM_2). Samples from the inner medulla were limited to the outer 4–5 mm and did not include the papilla. Immediately after dissection each sample was transferred to a preweighed counting vial, weighed again, and was then added 4 ml distilled water. The sample weights varied from 0.05 to 0.16 g. Equilibration of I-Ap and THO between tissue and water was found to be complete after 24 h. I-Ap and Ms activity was determined on the whole sample. Thereafter 100 µl of each sample was transferred to vials containing 6 ml scintillation fluid for measuring THO activity.

Blood samples of 0.1 to 0.3 ml for measurement of I-Ap activity were pipetted into preweighed counting vials, weighed again, and added 4 ml distilled water. To avoid cumbersome bleaching procedures, plasma instead of whole blood was used for determination of THO activity. One glass capillary was filled from each blood sample and spun in a hematocrit centrifuge. The plasma (about 0.02 g) was transferred to preweighed counting vials which were then weighed again and added 6 ml scintillation fluid. Calculation of whole blood THO activity was made on the assumption of uniform activity in plasma and red cell water using plasma and whole blood water content determined in each experiment (Clausen et al. 1979).

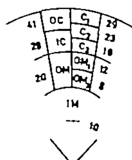


Fig. 2. Zonal division of kidney sector. The numbers give zonal volumes in per cent of total kidney volume.

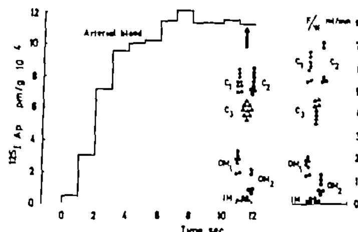


Fig 3 Arterial and tissue concentrations of I Ap in one experiment (Series A). Arrow indicates clamping of the renal pedicle relative to the arterial curve (corrected for catheter delay) Right Local blood flow calculated for individual tissue samples Zones defined in Fig 2

Microspheres Preparation and Injection

Two injections of Ms tagged with ^{85}Sr and ^{141}Ce (3M Co Minnesota) were made in the experiments of series B the second injection preceding I Ap and THO by 2–3 min Spheres with measured diameters of 16 1±2 4 (S D) μm were used in 7 expts spheres with diameter 1 9±1 3 μm in 3 expts. 4–800 000 spheres (5–10 μCi) were suspended in 1 0 to 1.5 ml 10% dextran sonificated and injected into the left ventricle in the course of 5 s A reference arterial blood sample was obtained from the femoral artery catheter by suction pump drawing 12.2 ml/min for a period of 45 s The number of microspheres trapped in a 100 mg cortical tissue sample was estimated at 100 to 250 corresponding to approximately 0 2 to 0 5 spheres per glomerulus.

Isotope measurements

Radioactivity was measured on the same day in tissue and blood/plasma samples as follows In series A and partition experiments ^{125}I-activity was measured in a single channel well scintillation counter In series B the acti ity of ^{125}I ^{141}Ce and ^{85}Sr was counted in a 3-channel well scintillation gamma counter (Searle Analytic Inc Model 1185) for 10 min (minimum 1 000 counts per cortical sample) Standards were included in each run to check window spillover ratios

Tritiated water activity was determined in a Mark I or Isocap 300 liquid scintillation system (Nuclear Chicago) using external standard to correct for quenching Interference by ^{125}I liquid scintillation was minimal because of the 10^3 times higher activity of THO in the infusate

Flow calculations

Average renal blood flow per gram tissue was calculated from the local flow rate and the respective zonal volumes. Cortex outer and inner medulla (including the papilla) were assumed to account for 70 20 and 10% of total kidney volume respectively The further subdivisions of the cortex were considered as 3 mm thick shells of an ellipsoide with diameters 7.3 and 4.3 cm (average for three kidneys of different size) giving the following volume fractions C 29% C_1 23% and C_1 18% Similar estimates for outer medulla gave 12% and 8% for OM_1 and OM_2 respectively (Fig. 2)

RESULTS

Series A

Series A represents our first measurements of I-Ap uptake in dogs and has several imperfections as compared to later experiments (Series B) The use of a short sampling catheter implies an unaccountable delay in the lower aorta and the timing and isotope counting were less accurate in some experiments While these shortcomings may have influenced absolute flow values the relative flow distribution should not be seriously affected The results are reported mainly because these experiments probably represent the best "control" flow distribution No experimental intervention was made on any kidney and the I Ap infusion was not preceeded by injection of microspheres which theoretically might influence flow distribution

Table 1 *Average coefficient of variation (per cent) of tissue sample tracer concentrations within zones*

Series A 9–10 samples from each zone in 9 kidneys
Series B 7–10 samples from each zone in 10 kidneys

Zone	Series A I Ap	Series B I-Ap	THO	Ms
C	11 1	4 9	11 7	16.2
C	12 7	6 7	10 6	18 4
C	16.5	12.2	17 3	33 1
OM	1.6	2. 1	4 1	69 8
OM	42.5	49 0	53.5	

able . Zonal blood flow per g tissue and zonal fraction of total renal blood flow determined by I Ap ean ± S.D

C, C_3=outer middle and inner third of cortex, OM OM$_i$=outer and inner half of outer medulla, IM inner medulla, =OM+IM DF=deep fraction=C_3+M

	Series A (9)		Series B (10)	
	Zonal flow (ml/min g)	Zonal fraction (%)	Zonal flow (ml/min g)	Zonal fraction (%)
	6 68_1.89	43 5±4.8	5 77±1 94	42.5±3.3
	6 50±1 60	33 5±1 4	5.57±1.87	32.6±1 7
	4.21±0.89	17.3_2.6	3 79±1 14	17 7_2.3
M	1 60±0 74	4 4±1 2	1 78±0 69	5.6±1 4
OM$_i$	0 57±0.38ᵃ	1.0±0.5	0 64_0.3ᵇ	1.3_0.4
M	1 14±0.41	5 4±2.5		
M	0 14±0 05	0.3±0 1		
I		5 9±2.3		7.2±1.8ᶜ
F		23 2±4 3		24 9±3 7
BF	4 45±1.01		3.90±1.25	

5 7 Mean of OM + OM$_i$ + IM in 5 expts. and OM + IM in 4 expts. Added 0.3% for IM

The effective I Ap infusion period defined as the ime from first appearance at the entrance of the ampling catheter to clamping the kidney varied rom 9 to 18 s (average 11 s) Mean arterial blood ressure ($\bar{AP}$) averaged 126 (S D 13) mmHg, and ematocrit was 38 6 (S D 4 6)

Primary data from one experiment are depicted n Fig 3 showing the arterial I Ap concentration curve and tissue concentrations in the various zones. For the sake of clarity the symbols representing concentrations in the different zones have been moved apart. Local blood flow calculated from the I-Ap concentrations of individual tissue samples are shown to the right in Fig 3 It is evident that the concentration pattern with about equal values in C and C_2, somewhat lower in C and sharply decreasing values in the medulla, is retained in the calculated flow However the nonlinear relationship between tissue concentration and blood flow results in greater scatter of flow rates in each zone and relatively greater differences between the various zones

The average coefficient of variation for I-Ap concentration within individual cortical zones ranges from 11 1 to 16.5% (Table 1) Since 9 to 20 samples were taken from each zone the SE of mean concentration will be 3–5% while calculated mean zonal flow will have a S E of 5–10% In three kidneys, one to three sectors had I Ap concentrations 20 to 50% lower than the average concentration of the remaining 7 10 sectors, but did not differ with respect to interzonal concentration ratios.

Calculation of zonal blood flow from mean zonal tracer concentration gives a lower value than the average of flows calculated from individual sample concentrations (Hope et al. 1976, Eklof et al 1974) However even with the rather large scatter observed in this series the average underestimate for cortical samples was only 1 6% Since even lower intrazonal variations were obtained in later expts. we find it justified to use mean zonal concentrations for flow calculation.

The zonal flow per gram tissue and its fraction of total renal blood flow are averaged for all 9 kidneys in Table 2, and Fig. 4 shows the zonal flow

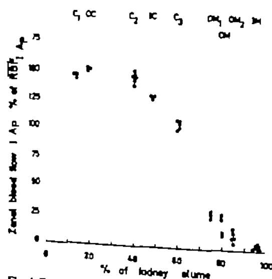


Fig 4 Zonal flow estimated from I-Ap uptake in 9 kidneys (Series A) in per cent of individual mean RBF_{I-Ap}. Zones defined in Fig. 2

Table 3 *Zonal blood flow per g tissue and onal fraction of total renal blood flow determined in 10 control kidneys (Series B) with microspheres (Ms) ¹²⁵I Iodoantipyrine (I Ap) and tritiated water (THO) Mean ± S.D*

	Ms		I Ap		THO	
	Zonal flow (ml/min g)	Zonal fraction (%)	Zonal flow (ml/min g)	Zonal fraction (%)	Zonal flow (ml/min g)	Zonal fraction (%)
C	6 34±2 42	50 8±7.5	5 77±1 94	4 .5±3.3	5.31± .57	40 3±3 7
C	5 42± .50	33.3±4 6	5.57±1 87	32 6±1 7	5 4_±_.59	3_9± [illegible]
C	? 89±1 66 *	13 7±3 8	3 79±1 14	17 7±? 3	3 91±1 79	18 8±2 4
OM	0 63±0.57* *	1 9±1 1	1 78±0 69	5 6±1 4	1 97±1 15	6_±1 7
OM	0 17±0 14 *	0 4±0._	0 64±0.3	1 3±0 4	0 68±0 3?	1 5±0 5
RBF	3 94±1.53		3 90±1 25		3 78±1 78	
DF		16 0±4 8		4.9±3 7		26 8±4

Significantly different from THO Wilcoxon signed rank test for paired samples Abbreviations as in Table
Significantly different ($P<0$ 05) from I Ap

in individual expts in per cent of average flow per gram kidney The flow distribution showed no relationship to the duration of I Ap infusion (9–18 s) or to the degree of saturation of C (30–7? %) (Degree of saturation calculated as tissue concentration/λ_{tis} for I Ap divided by the average concentration of the last three one second blood samples)

Total renal blood flow averaged 4 45 (S D 1 01) ml/min g with a range of 3 19 to 5 94 ml/min g

Series B

As described in Methods Series B provided a direct comparison of blood flow measured in the same tissue samples with Ms and within 2–3 min with I Ap and THO Acetylcholine was infused continuously into one renal artery raising total RBF_{emf} (electromagnetic flowmeter) of the infused kidney by 50 to 100% above control level Because of the high flow the effective infusion time for I Ap/THO was reduced to average 10 3 s (8 to 13.5 s) $\overline{AP}$ averaged 126 mmHg (S D 15) and Hct 38 0 (S D 6 6) A moderate (<15 %) and shortlasting fall in arterial pressure and RBF_{emf} of the acetylcholine infused kidney was occasionally observed after Ms injection but at the time of I Ap/THO infusion RBF_{emf} averaged 98% of preinjection control Data for intrarenal flow distribution are given only for the control kidney whereas calculated total RBF is reported for both kidneys

Flow distribution within each one As evident for intrarenal flow distribution [are] given only for the control kidney whereas calculated total RBF is the scatter was considerably lower than in Series A and a coefficient of variation of less than 25 was observed in C and C_2 in 5 expts. Considerably greater scatter was observed for THO concentrations within each zone and without exceptions Ms activity showed the greatest variation (Table 1) However the comparison of concentrations may be somewhat misleading since in the case of Ms the relative scatter for estimated flow will be identical to that of the tissue concentrations whereas in the case of I Ap and THO the coefficient of flow variations among samples will be higher than that of the tissue concentrations because of the nonlinear relationship between tissue concentrations and estimated flow (Fig 4) Thus the coefficient of variation for I Ap flow will be about 8% in C_1 and C_2 and about 15% in C_3. The corresponding numbers for THO are 16 and 3 Since an average of 9 samples were taken from each zone the standard error of mean zonal flow estimated with I Ap will be about ? 7% in C_1 and C_2 and 5% in C_3 Corresponding numbers for THO are 5 and 8% and for Ms 5.5 and 11% As evident from Table 1 a much greater scatter was obtained in the medullary zones with all indicators

In one kidney two sectors had indicator concentrations ?0–30% below average

Flo di tribution bet een ones Total RBF estimated from I Ap varied considerably among experiments (Fig 7) with an average about 12% lower than that obtained in Series A (Table 2) The difference reflects lower flow in all cortical zones ($P>0$ 05) whereas little difference was observed in outer medulla The zonal flow fractions showed less variation and were quite similar in the

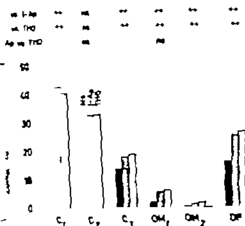


Fig. 5. Zonal flow in per cent of RBF measured with Ms (black columns), I-Ap (hatched columns) and THO (clear columns). Average of 10 control kidneys (Series B). Deep fraction, DF: Medulla + C_4. Zones defined in Fig. 2. Significance levels: $P<0.02$, $P<0.05$, ns $P>0.05$.

two series, except for somewhat lower ($P>0.05$) outer medullary fractions in Series A (Table 2).

As evident from Table 3 and Fig. 5 the average zonal flow and flow fractions obtained with THO were quite similar to that obtained with I-Ap except for slightly higher C and outer medullary flow fractions with THO.

A clearly different pattern was observed with Ms. As expected, medullary Ms-activity was low but clearly indicated the presence of microspheres in most tissue samples from OM, in some samples from OM_2, and even in occasional IM samples. In addition the flow fraction in C_4 was about 25% lower with Ms than with I-Ap and THO. A significantly higher flow fraction was obtained with Ms in C, whereas the three indicators agreed well in middle cortex C_3. As will be discussed later the deep flow fraction, DF, defined as the fraction of total RBF going to C_4+medulla may provide a useful parameter for comparison. DF averaged 16.0% for Ms compared to 24.9 and 26.8% for I-Ap and THO respectively. As shown in Fig. 6, DF_{THO} and DF_{I-Ap} agreed well and in spite of considerable scatter DF_{Ms} was lower in all expts. The difference to DF_{I-Ap} and DF_{THO}, averaging about 0.09 and 0.11 respectively, seemed independent of the magnitude of DF and approximately the same difference was maintained also when comparing DF: C_4 (Fig. 8).

The distribution of Ms with diameter 12.9±1.3 µm (3 expts.) did not differ from that observed with diameter 16.1±1.4 µm.

Inner medullary I-Ap and THO uptake was usually not measured in this series because the infusion time was too short for a meaningful calculation of inner medullary flow. (In calculating DF (Table 3, Figs. 5 and 6) the average value of 0.3% of RBF observed in Series A was used.)

Total renal blood flow. The average RBF calculated from the three indicators were not significantly different in the control kidney (Table 3) nor in the acetylcholine infused kidney. In the latter the following means were obtained: I-Ap 6.40 (S.D. 1.96), THO 6.35 (S.D. 2.30) and Ms 6.72 (S.D. 2.1) ml/min g. With some exceptions, the agreement was good also in individual experiments, but as shown in Fig. 7 there was a slight tendency for $\overline{RBF}_{I-Ap}$ and $\overline{RBF}_{THO}$ to fall off relative to $\overline{RBF}_{Ms}$ with increasing flow. However the slope of the regression lines did not deviate significantly from unity ($P>0.05$).

DISCUSSION

Validity of the Kety model and the tissue sampling technique

A large number of elaborate mathematical models have been designed to describe the blood tissue

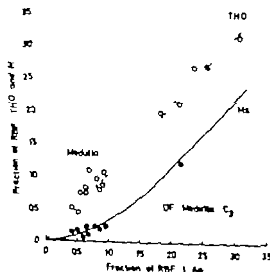


Fig. 6. Fractions of total renal blood flow to medulla and to medulla + C_4 (DF), as estimated by THO (open circles), Ms (closed circles) and I-Ap in 10 control kidneys (Series B). Identity line broken. Solid line representing Ms vs. I-Ap drawn to best visual fit.

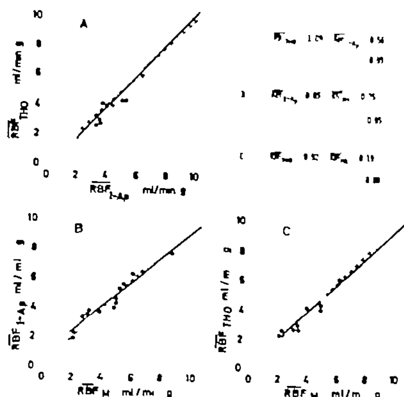


Fig 7 Comparison of mean RBF in 70 kidneys as measured simultaneously with THO I Ap and *Ms (Series B). Open circles, Acetylcholine infused kidneys. Filled circles:* Control kidneys Identity lines broken, regression lines solid. Regression coefficient not different from 1 00 ($P>0.05$)

exchange of inert diffusible substances However for the purpose of estimating local blood flow from tracer concentrations in tissue and arterial blood the simple exponential model proposed by Kety (1951) would still seem unsurpassed Two main assumptions will be briefly discussed with special reference to the kidney (1) A correct estimate of total renal blood flow requires diffusion equilibrium between each region of the kidney and venous blood leaving that particular region (2) A true es-

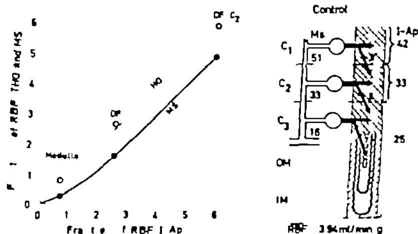


Fig 8 Left Average flow fractions in medulla, medulla+C (DF) and DF+C as estimated with THO (open circles) M (filled circles) and I Ap I 10 control kidneys (Series B) Identity line broken Solid line representing Ms vs. I-Ap drawn to best visual fit *Right* Hypothetical postglomerular inward flow of blood to account for the difference between zonal flow calculated from Ms and diffusible tracers in control kidneys The numbers give flow in per cent of total RBF

mate of regional blood flow requires in addition that the inert tracer is delivered simultaneously to each tissue region exclusively by arterioles/capillaries of that particular region

The first assumption, venous equilibrium with the tissue is in all probability not satisfied for the medulla. Due to counter current diffusion between arterial and venous vasa recta, blood leaving the medulla during tracer uptake will have a higher tracer concentration than the average concentration in the medulla. The result is an underestimate of blood flow in the medulla as whole and more so the higher the diffusibility of the tracer In an attempt to elucidate this point THO was added to the I-Ap infusion in Series B based on the assumption that THO should have a lower diffusion coefficient (D) in tissue than the lipid soluble I-Ap. Evidence for such relationship has been presented for D_2O versus unlabeled antipyrine in the liver (Thompson et al. 1959)

The lack of direct measurements of $D_{I\text{-}Ap}$ and D_{THO} in the kidney prompted the following indirect, and approximate estimates As Vaupel (1976) recently deducted from large number of literature data, the tissue diffusion coefficients of the 4 gases O_2, CO_2, N_2 and H_2 are exponentially related to tissue water content In the kidney with water content of 85 % the diffusion coefficients should be about 50 % of the free diffusion coefficients in water Assuming the same relationship for I-Ap, and using Graham law the $D_{I\text{-}Ap}$ for the kidney at 37°C may be calculated from Vaupel's data: For instance, from D_{N_2} is obtained

$$D_{I\text{-}Ap} = D_{N_2}(MW_{N_2}/MW_{I\text{-}Ap})^{0.5} = 1.4 \times 10^{-5}(28/315)^{0.5} = 4.2 \times 10^{-6}\ cm^2$$

Similarly from H_2, O_2 and CO_2, $D_{I\text{-}Ap}$ values of 2.4 10^{-6} 4.8 10^{-6} and 5.4 10^{-6} cm² are obtained Disregarding the discrepant value obtained from D_{H_2} $D_{I\text{-}Ap}$ of about 5 10^{-6} cm² would seem to be reasonable estimate

For THO Chinard et al (1969) estimated from renal venous dilution curves kidney diffusion coefficient of 18 % of that in water about 6 10^{-6} cm² $^{-1}$ This estimate was based on comparison with Hexanol and the assumption that $D_{hexanol}$ is the same in kidney tissue as in water If however $D_{hexanol}$ has the same dependence on tissue water content as the gases mentioned above D_{THO} would come out as 3 10^{-6} cm² A similar value may be estimated from Fig 7 in the same study (Chinard et al 1969), showing D_{THO} of about 40 % of $D_{Cr\text{-}EDTA}$. Comparable values may also be estimated from microppuncture studies on tubular THO permeability in rats The data of Persson (1970) and Persson & Ulfendahl (1970) on permeability and an estimated tubular wall thickness of 10 μm give D_{THO} for proximal tubules of 5.6×10^{-6} for distal tubules 3.2 10^{-6} with ADH present, and 1.6×10^{-6} cm²·s⁻¹ without ADH

In conclusion, the diffusibility of I-Ap and THO in the kidney would seem to be quite similar both having a D of 3–5×10^{-6} cm² s This agrees well with the similar uptake rates for I-Ap and THO in the inner medulla observed in partition experiments (Clausen et al 1979) indicating that AV shunting in the vasa recta bundles affects the two tracers to the same extent

It follows that the simultaneous determination of I Ap and THO does not provide any further insight in the medullary counter current exchange It must be assumed, however that both substances will underestimate medullary flow to an unknown extent. Because the medullary flow fraction by all estimates is small this conclusion is not incompatible with the quite good agreement between total renal blood flow estimated from THO I-Ap and Ms. On the other hand the agreement on total flow provides strong support for the assumption of venous blood/tissue equilibrium and therefore flow-limited uptake for both THO and I Ap in the renal cortex even at near maximal vasodilation (Fig. 7). Still, both I-Ap and THO could violate the second requirement set out above i. e that the tracer should be delivered exclusively by local capillaries If for instance appreciable amounts of tracer are delivered to the deep cortex by diffusion from the interlobular arteries capillary blood flow in this zone would be overestimated It would follow that outer cortex were fed by blood having lower tracer concentration than arterial blood leading to an underestimate of outer cortical capillary flow It should be realized that a diffusion bypass" (Chinard et al. 1969) of this type does not in itself violate the assumption of venous blood/tissue equilibrium, and is therefore not excluded for either I Ap or for THO by the good agreement with total flow calculated from Ms uptake On the other hand this agreement does speak against an appreciable direct arteriovenous diffusion shunt, which is also unlikely for anatomical reasons: While there is close AV proximity for the larger vessels (interlobar and arcuate) interlobular arteries and veins seem to run more separately (von Kügelgen et al. 1959 Barger & Herd 1973).

Tracer leaves the blood by diffusion from the peritubular capillaries and—at the same time—by glomerular filtration The cortical tissue consisting mainly of tubules and their content, will thus be fed from "two sides This might to some extent help to ensure complete tracer equilibration but since

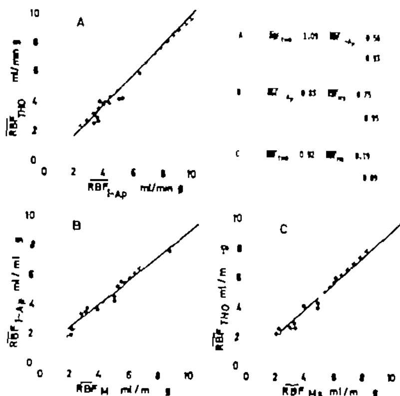


Fig 7 Comparison of mean RBF in 20 kidneys as measured simultaneously with THO I Ap and Ms (Series B) Open circles Acetylcholine infused kidneys Filled circles. Control kidneys Identity lines broken regression lines solid Regression coefficient not different from 1 00 ($P>0\ 05$)

exchange of inert diffusible substances However for the purpose of estimating local blood flow from tracer concentrations in tissue and arterial blood the simple exponential model proposed by Kety (1951) would still seem unsurpassed Two main assumptions will be briefly discussed with special reference to the kidney (1) A correct estimate of total renal blood flow requires diffusion equilibrium between each region of the kidney and venous blood leaving that particular region (2) A true esti-

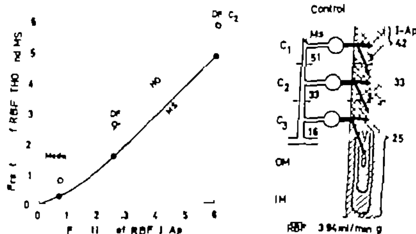


Fig 8 Left Average flow fractions in medulla, medulla + C_3 (DF) and DF + C as estimated with THO (open circles) Ms (filled circles) and I-Ap in 10 control kidneys (Series B) Identity line broken Solid line representing Ms vs. I Ap drawn to best visual fit *Right* Hypothetical postglomerular inward flow of blood to account for the difference between zonal flow calculated from Ms and diffusible tracers in control kidneys The numbers give flow in per cent of total RBF

10 to 20 samples in each zone are averaged the standard error of the mean zonal flow (5.5% in C_1 and C_2, 11% in C_3) is well acceptable for comparison of flow estimated with Ms to that obtained with I Ap and THO

All indicators showed much greater intrazonal scatter in C_3 and the medullary zones than in C_1 and C_2. This does not necessarily signify a greater flow heterogeneity but rather results from the steep concentration (and flow) gradient in this region (Fig. 4), together with the problem of obtaining a consistent zonal division. Even with careful dissection under low power magnification, performed by the same person there was evidence for considerable variation in dissection both within a single kidney and among different kidneys. Appreciable numbers of Ms might occur in several OM samples and even in OM samples while most samples from these zones were completely free of Ms. These observations emphasize the hazard of comparing results obtained at different laboratories and indicate that reliable comparison of two tracers demands determination of both tracers in the same tissue samples

Sectors with low flow 20–50% below average as estimated with all indicators were occasionally observed While no specific faults were detected in these experiments we tend to believe that the phenomenon represents an experimental artifact rather than physiological flow heterogeneity

Flow distribution between zones

Comparison to other diffusible tracers As already pointed out above comparison of data from different laboratories should be made with caution because of the lack of uniform procedures for zonal division of the kidney In the case of H washout studies made in this laboratory comparison seems reasonably safe While the medullary flow patterns are quite similar (Aukland 1967) H measurements tend to give less difference between outer and inner cortical flow than observed with I Ap and THO (Aukland et al 1973) The difference might result from the greater diffusibility of H_2 causing greater exchange by the interlobular arteries in inner cortex

In the case of ^{85}Kr autoradiograms suggest a much lower flow in deep (juxtamedullary) than in superficial cortex (Barger and Herd 1973) However the only quantitative local measurements of ^{85}Kr recently presented by Passmore Neiberger and Eden (1977) rather suggest a similar initial washout rate throughout the cortex. Since ^{85}Kr also is more diffusible than I Ap and THO (Chinard et al. 1969) the explanation might be as suggested for H None of these studies would seem to fit well with the common compartment analysis of externally recorded ^{85}Kr-washout indicating that inner cortex and outer medulla can be represented by a common desaturation rate about one third that of outer cortex (Thorburn et al 1963 Barger & Herd 1973) Outer medullary flow calculated from [I Ap] and THO agrees well with that obtained from ^{85}Kr by Passmore et al. (1977) but are higher than the few in situ measurements reported by Aukland & Wolgast (1968)

Uptake of ^{86}Rb which depends on the sink effect of the large intracellular potassium pool, gives a cortical flow distribution close to that obtained with I Ap and THO (Bálint Bartha & Fekete 1969) However ^{86}Rb shows a relatively much greater uptake in the medulla Two explanations would seem likely (1) Less counter current exchange of the lipid insoluble ^{86}Rb, or (2) higher extraction of ^{86}Rb in the medulla than in cortex.

In conclusion While the flow distribution pattern obtained from I-Ap and THO uptake is basically similar to that reported for other diffusible tracers, there are discrepancies which in part may be due to differences in diffusibility

Comparison of diffusible tracers and microsphere uptake When comparing zonal flow estimated from diffusible tracers versus microsphere uptake one should keep in mind the different ways of uptake While practically all Ms (15 μm) are arrested in afferent arterioles or glomeruli (McNay & Abe 1970, Ofstad Willassen & Egenberg 1973) and therefore reflect glomerular blood flow the diffusible tracers are probably delivered mainly by the postglomerular peritubular capillaries Thus the low Ms uptake in the medulla is a simple consequence of the absence of glomeruli in this region However if the glomeruli in C_3, the inner third of the cortex deliver all blood flow to the medulla through the vasa recta, and in addition all blood flow to the peritubular network in C_3, the Ms flow in C_3 should equal I-Ap/THO flow in C_3 + medulla. This was not the case in any experiment. Ms flow to C_3 was in fact lower than I Ap and THO flow in C_3 alone in all 10 kidneys. Since total flows showed good agreement it follows that there must be a surplus of Ms relative to I-Ap/THO flow in

the convoluted proximal tubules are located close to their glomeruli filtration per se will probably not influence the location of tracer uptake (See below)

The requirement of simultaneous delivery of tracer to the exchange vessels seems to be well satisfied for the cortex An estimated passage time through the interlobular arteries of 0 1 s (probably overestimated) leads to an underestimate of outer cortical flow by only 1 %

As discussed previously (Hope et al 1976) the presence of unequilibrated arterial blood in the kidney results in overestimation of blood flow approximately corresponding to its fraction of kidney volume i e only a few per cent Similar calculations show that the same applies to venous blood present in non-exchange vessels No appreciable influence on zonal flow ratios would be expected

Even if the deviations from the theoretical requirements are considered acceptable the practical problems in determining local uptake rate by the tissue sampling technique are considerable

Since the high cortical blood flow and rapid tracer uptake necessitate a short infusion period the timing of the arterial concentration curve to the moment of renal circulatory arrest is critical As described under Methods (Fig 1) the transit time through the terminal aorta may if not accounted for cause up to 15% overestimate of cortical blood flow The somewhat higher flow observed in Series A than in Series B may at least in part be due to such errors Obviously a shortening of the infusion period will increase the effect of timing error and will also preclude measurement in low flow regions On the other hand prolongation of the infusion period with a higher degree of saturation will increase the error in calculated flow caused by an inappropriate λ_m and by inaccuracies in the measurement of tracer concentrations in blood and tissue (Hope et al 1976 Eklöf et al 1974) Thus in the experiment shown in Fig 3 with a I Ap saturation of about 50% in C a 10% error in λ_m (~one S D in our estimate of λ_m for I Ap (Clausen et al 1979)) will give an error in calculated flow of only 5% In another experiment with a saturation of 72% which was the highest level accepted a 10% error in λ_m would lead to 15% error in calculated flow In the latter case the deep flow fraction would be in error by 1.5% of total RBF for instance 21.5% instead of 20%

As described elsewhere I Ap infusion periods of 75–300 s resulted in higher values for flow in IM (Avg 0 74 ml/min g) than that observed in Series A (Clausen et al 1979) This finding corroborates previous results showing a considerable initial delay in H_2 washout in IM (Aukland & Berliner 1964) Thus short infusion periods suitable for the cortex consistently cause underestimation of IM flow with diffusible tracers Although prolonged infusion minimizes the error due to the initial delay it does not prevent underestimation of flow due to countercurrent exchange of tracer (Hope et al 1976)

Since we found no practical way to determine λ_m for I Ap in the medulla we have assumed a value equal to that determined for the cortex This is not quite satisfactory but the error in the medullary flow estimate resulting from an inappropriate λ_m should be small because of the low degree of saturation (well below 50%) and probably of minor importance compared to the error mentioned above The true total medullary blood flow is presumably considerably higher than that obtained with I-Ap and THO (or with other diffusible tracers) but any underestimate will only strengthen the conclusion drawn from the comparison with Ms (see below)

While the theoretical and practical problems discussed above may introduce considerable errors in absolute flow estimates none of them are likely to cause great errors in the flow ratios between the various cortical zones

Flow distribution within each zone

The small scatter of I Ap concentrations within cortical zones C and C_3 (coefficient of variation as low as 2 5 in some expts average 4 9 and 6.7% in C and C respectively) indicates a remarkable intrazonal flow homogeneity among tissue regions of 50 to 150 mg A rough estimate of the analytical errors involved suggests that the true coefficient of variation for cortical concentrations is on average less than 3% corresponding to a coefficient of variation for blood flow of 5% or less

The much greater scatter of cortical THO concentration is probably due to less acurate determination of β-activity While in theory the two tracers might be equally well suited for flow measurement and have been shown to give practically identical average results there is no doubt that in our hands the I Ap is the most reliable

The greater intrazonal scatter of Ms-activity (Table 1) is explained by the rather small number of spheres in each tissue sample (100–250) (Buckberg et al 1971) However when the activities of

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other parts of the kidney As evident from Fig 5 good agreement was found in C_4 while the Ms surplus was located in C_1 This conspicious and consistent disparity might result from one or several of the following mechanisms

(1) Microspheres are *underrepresented in deep* glomeruli relative to glomerular blood flow This might be caused by axial streaming by a streamline effect (Svanes & Zweifach 1968) or simply by steric exclusion by narrow afferent aterioles (Morkrid Ofstad & Willassen 1976) According to previous studies in dogs (Slotkoff et al 1971 *Mimran Guiod & Hollenberg 1974*) there should be no size-dependent maldistribution of spheres with the average size and the narrow diameter range of the Ms-batches used in the present study However available data would not seem to exclude skimming with little or no size-dependency i e even with complete uniformity of Ms diameters deep cortical flow might be underestimated

(2) Too much I Ap and THO are deposited in deep cortex and medulla relative to glomerular flow in this region

(*a*) If appreciable diffusion of the tracers takes place across the interlobular arteries or if

(*b*) a considerable fraction of flow in the peritubular network in one cortical layer is derived from glomeruli situated in more superficial layers as previously proposed for the rat kidney (Hope et al 1976)

A model based on the assumption that mechanisms 1 and 2*a* are not at work suggests an inward cortical postglomerular flow component corresponding to 9% of total RBF (Fig 8) A net inward flow of tubular fluid would have a similar effect However it may be calculated from the data of Nissen (1966) on the cat kidney that the inward tubular flow could maximally account for one third of the observed disparity between Ms and diffusible tracers An estimate based on the inward directed flow in the straight segment of the proximal tubules leads to the same conclusion With a total blood filtration fraction of 0 15 and 60% reabsorption in the proximal convolution the straight segment flow would be 6% of local flow In comparison the estimated I Ap flow from C to C_4 corresponds to 18% of flow in C_1 (Fig 8) Furthermore since the tracer will tend to *equilibrate with surrounding tissue en route* to deeper regions the actual amount of fluid crossing the zonal borders would probably have to be considerably greater Inward transport of more diffusible tracers for instance H_2 and ⁸⁵Kr might accordingly be smaller

While admittedly speculative the last explanation (2*b*) might be the most attractive but the present experiments do not rule out maldistribution of *Ms or interlobular artery exchange of diffusible* tracers Further discussion should therefore await more experimental data

This work was supported in part by grants from "Eva Langfeldts Fond" and from The Norwegian Research Council for Science and the Humanities.

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he effect of transmural field stimulation on the erotonin content in rat duodenal enterochromaffin ells—in vitro

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PETTERSSON G AHLMAN H DAHLSTRÖM A KEWENTER, J LARSSON J & LARSSON P A The effect of transmural field stimulation on the serotonin content in rat duodenal enterochromaffin cells—in vitro. Acta Physiol Scand 1979 107 83–87 Received 23 March 1979 ISSN 0001-6772. Institute of Neurobiology and Department of Surgery III, University of Göteborg Sweden.

The effect of transmural field stimulation (TFS)—*in vitro*—on the serotonin (5-HT) content in enterochromaffin cells (EC) in rat duodenum was studied with cytofluorimetric method TFS caused significant 25% decrease of 5-HT in EC. The presence of tetrodotoxin or d l-propranolol in the stimulation bath antagonized the effect of TFS In biopsies from rat pretreated with 6-OH-dopamine TFS had no effect of the 5-HT content in EC The results of the present study strongly suggest that the TFS induced decrease in 5-HT content is due to direct neural, probably β-adrenoceptor mediated influence on the EC

Key ords Rat enterochromaffin cells, duodenal mucosa, field stimulation, cytofluorimetry serotonin release adrenergic mechanism

The serotonin (5-HT) containing enterochromaffin cell (EC) is a basal-granulated gut endocrine cell with amine handling properties according to the APUD concept (Pearse 1969). Different populations of EC may store polypeptides beside the amine e g. motilin, substance P or enkephalin (Pearse et al. 1974 Pearse & Polak 1975 Polak et al 1976, Alumets et al 1978)

EC are predominantly of the open type i.e. with a brush of microvilli protruding into the gut lumen (Fujita & Kobayashi 1971) Luminal stimuli e.g. acid pH hypertonic sugar solution and an increased intraluminal pressure may cause degranulation of the cells and increased levels of 5-HT in the portal blood or in the lumen (Resnick & Gray 196.. Kobayashi & Fujita 1973 O'Hara et al 1960 Drapanas et al 196.., Bulbring & Crema 1959). The parallel decrease of 5-HT content in EC and increase of portal 5-HT suggest that the amine is released from the EC upon stimulation Also neurogenic release mechanisms have been suggested for 5-HT since stimulation of the splanchnic as well as of the vagal adrenergic nerve fibers to the gut causes similar changes (Burks & Long 1966a Tansy et al 1971 Ahlman et al 1976a). Results from cytofluorimetric studies of rat duodenal biopsies incubated in vitro with adrenergic agonists and antagonists indicate that at least a β-adrenoceptor mechanism is involved in the release of 5-HT from EC (Pettersson et al. 1978)

In vitro methods offer several advantages in the study of receptor and transmission mechanisms, since (*a*) a well defined external environment regarding e g pH ionic composition and drug concentrations can be established and (*b*) secondary effects from changes in secretion and regional blood flow caused by nerve stimulation and drugs do not influence the results. Transmural field stimulation (TFS) of isolated tissues in vitro causes an activation of nerve fibers within the specimen (Paton 1955 Farnebo 1970)

The aim of the present investigation was to fur

able 1 *The effect of TFS (18 V 2 ms 10 Hz, 15 min) on the 5-HT content in duodenal EC from normal ts and rats pretreated with 6-OH DA*

ean value and S E. of 20 cells from each specimen of each individual rat is shown. Difference from incubated unstimulated control indicated by* ($P<0.01$). Original values given within brackets to illustrate variation in fluorescence yield irrespective of simultaneous tissue processing

ormal rats		6-OH DA rats	
nstimulated	Stimulated	Unstimulated	Stimulated
00±7.3 (525±38)	47±3.2**	100±4 4 (566±25)	89±4 4
00+7.9 (446±35)	72±3.6**	100±5 7 (509±29)	81±4 7*
00±6.5 (385±25)	73±4 4**	100±5 6 (387±22)	98_3.6
00 6 1 (400±.4)	64±4.2**	100±7 7 (170±13)	104_6 4
00±9 1 (486±44)	73±6.7**	100±8 7 (137±12)	96±6 3
00±4 3 (540±23)	79±3.0**	100±6.1 (180±11)	107±8.3
00±5.5 (396±22)	101±7.8		
00±2 2 (168± 4)	64±5 7**		
00±2 8 (175± 5)	92±5 4		
00±5 4 (197±11)	82±5.8**		
00±7.2 (161 12)	89±7.9		
00±6 0 (197±12	62±3.6**		
Mean 100±1 7	75±4 3*	100±2.6	95_3.9

was no obvious decrease in the fluorescence intensity of the adrenergic nerve terminals

When *tetrodotoxin* (10^{-6} M) was added to the stimulation medium no significant change in EC fluorescence was demonstrated in 5/6 animals (Table 2). The contractile response at the TFS was minimal or absent with tetrodotoxin in the bath but the procedure was judged effective by the appearance of gas at the electrodes. *Propranolol* (10^{-6} M) in the medium had a blocking effect on the decrease of 5-HT in EC (Table 2) but did not block the motor response The blocking effect of tetrodotoxin and propranolol in the bath was statistically significant ($P<0.05$) as compared to TFS without drugs added.

In the gut specimens of the *6-OH-DA pretreated* rats TFS had no effect in 5/6 animals (Table 1). In these specimens no adrenergic terminals could be observed in the mucosa and submucosa. The almost complete absence of adrenergic nerve terminals was also controlled in stretch preparations of irides from these animals. A normal motor response was obtained.

Table 2 *The influence f tetrodotoxin and propranolol on the effect of TFS on the 5-HT content in duodenal ED*

The ratio stimulated/unstimulated is given for each rat. Difference from specimens stimulated in Krebs' solution only indicated by* ($P<0.05$)

Krebs*	Tetrodotoxin 10^{-6} M	Propranolol 10^{-6} M
Mean 0 75±0 04	0 91±0 02*	0.93±0 02*

See Table 1 *Normal rats

DISCUSSION

From nerve *stimulation* expts in vivo there is evidence for a neurogenic release of 5-HT from EC into the portal stream and possibly also to the gut lumen (Ahlman et al 1978) However such stimulation of the vagal and splanchnic nerves had profound effects on the motility blood flow and secretion of the intestine: effects that may influence the functional state of the EC. Burks and Long (1966*b*) reported that stimulation of intestinal motility caused release of 5-HT from the gut. Therefore when testing the hypothesis of a neurogenic release of 5-HT from EC, in vitro techniques may offer an advantage being test systems without most of these secondary effects On the other hand the biopsies are then transferred from their natural environment to artificial media and long neuronal pathways are interrupted.

The TFS procedure used in the present study caused a well reproducible decrease of the 5-HT

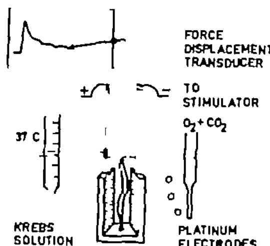


Fig 1 Experimental model The gut strip was mounted between two parallel platinum meshwork electrodes 7 mm apart in an open plexiglass chamber containing oxygenated Krebs solution (37°C) with the drug to be tested The lower end of the specimen was fixed to the bottom of the chamber and the upper end connected to a force displacement transducer The electrodes were connected to a stimulator

ther study the neural influence on the EC with special regard to adrenergic components using TFS

MATERIAL AND METHODS

30 Sprague Dawley rats (males 250 g) were killed by decapitation The proximal 5 cm of the duodenum was dissected free and placed in icecold Krebs solution. The gut was then cut into 12×6 mm² longitudinal strips including all 3 layers of the wall Each strip was then divided into two longitudinal halves, one half to be stimulated and the other to serve as a control The strip to be stimulated was mounted between two parallel platinum meshwork electrodes (7×1 mm²) 7 mm apart in an open plexiglass chamber (Fig 1) The lower end of the specimen was fixed to the bottom of the chamber and the upper end connected to a force displacement transducer to record the contractile strength developed at TFS The stimulation bath contained Krebs solution (37°C oxygenated with 5% CO_2 95% O) with the drug to be tested Each control strip was incubated simultaneously in the same medium during the same period of time as the stimulated strip The specimens were kept in the medium for equilibration 10 min before stimulation The stimulation was given from a Grass S9 stimulator using the following parameters 18 V 2 ms 10 Hz during 15 min. The current through the bath was 5–6 m A/mm ms. The stimulation procedure was judged to be effective by a contractile response from the specimen and by the development of free gas at the electrodes

Drugs The drugs used for incubations were tetrodotoxin 10^{-6} M (Sigma chem.) and d 1-propranolol 10^{-6} M (Inderal® ICI) 6 rats were pretreated with 6-OH dopamine (Sigma chem) 100 mg/kg b wt i p 48 h before

the expts No further drug incubation with strips
these animals were done.

Histochemistry and cytofluorimetry After incub
and stimulation the gut specimens were immedi
frozen in liquid propane freeze-dried and treated
formaldehyde vapour generated from paraformalde
for fluorescence microscopy according to the Falck
arp technique (for description and references see C
& Jonsson 1967) The cytofluorimetric method ha
described in detail earlier (Ahlman et al 1976)
cytofluorimetry was performed with a Leitz
photometer with an illuminator for incident
positioned to isolate the activation maximum of the
fluorophore at 410 nm The measuring diaphragm
fixed at a diameter of about 20 μm enough to encom
the longitudinally cut EC. From each specimen the
fluorescence intensity of 20 EC was recorded.
relative measure of the 5-HT level of each EC,
fluorescence intensity after 40 s of ultraviolet illumi
was recorded For each cell the background fluoresc
was checked separately and subtracted from the mea
intensity of the cell These values were expressed in
trary units and fed into a Hewlett Packard 9821A co
ter system The mean value of intracellular 5-HT flu
cence intensity in each control i.e the section from
parallel-incubated unstimulated strip, was set to 10
The mean value and S E for each stimulated speci
was then compared to its unstimulated control Stud
t-test was used for comparisons within each animal
coxon s test for paired and unpaired data were used
comparisons within and between groups of animals
P-value <0 05 was regarded as significant The freq
cy-distribution of the fluorescence intensities rece
from each sample was normal

All specimens from one animal were processed
taneously and all measurements were performed by
same person under identical conditions using coded
ples In order to standardize the cytofluorimetric m
urements the following criteria were used. (1) Speci
must have no obvious amine diffusion in adrenergic te
nals or EC (2) EC must be sectioned through the b
lamina the nucleus and the luminal process. (3) Only
located in the crypts were measured (4) In each fiel
view the EC of medium fluorescence intensity (subjecti
ly judged), fulfilling criteria 1 3 was selected for me
urement

RESULTS

Nonstimulated specimens either incubated
Krebs solution only or with any of the dr
added demonstrated distinct beaded adrener
nerve terminals with blue-green fluorescence
strongly yellow fluorescent FC

TFS caused a significant 25% decrease of t
intracellular 5-HT fluorescence after 15 min
stimulation demonstrated by cytofluorimetry (T
ble 1) The morphology was well preserved The

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content in EC Addition of tetrodotoxin which in hibits the propagation of action potentials in the nerves (Kao 1966) minimized the motor response and antagonized the effect of TFS on the 5 HT content in EC Thus the effect of TFS is mainly mediated through activation of nerves within the specimens and not a direct activation of the EC

The β adrenoceptor antagonist propranolol had a blocking effect equal to tetrodotoxin on the 5 HT decrease in EC upon TFS This effect of propranolol may be a true β-adrenoceptor blockade or be due to its membrane stabilizing effect or possibly a combination of these mechanisms However in biopsies from rats pretreated with 6-OH DA which causes a selective chemical destruction of adrenergic nerve terminals (Malmfors and Sachs 1968) no change in intracellular 5 HT levels could be demonstrated after TFS This gives further support to the hypothesis of an adrenergic probably β-adrenoceptor mediated release of 5-HT from EC upon TFS Since the motor responses in the propranolol and 6-OH DA groups remained unchanged (as was the 5-HT content in EC) the motor activity per se could not be responsible for the release of 5-HT upon the TFS In a previous in vitro study where rat duodenal biopsies were incubated with various tentative transmitters it was found that not only adrenergic agonists (adrenaline noradrenaline and isoprenaline) but also acetylcholine caused a decrease of 5-HT in EC (Pettersson et al 1978) In the rat a possible cholinergic release of 5 HT from EC has been suggested also in vivo since the decreased tissue concentration in the intestine and elevated portal levels upon vagal nerve stimulation could be partially blocked by atropine (Tobe et al 1976) Thus also neural mechanisms other than an adrenergic mechanism may possibly influence the EC From a morphological point of view the EC may be influenced by several types of nervous elements Ultrastructural studies have demonstrated autonomic nerve endings of cholinergic adrenergic and peptidergic types in very close relation to the base of duodenal EC in the guinea pig (Lundberg et al 1978)

Previous studies (Burks & Long 1966a Ahlman et al 1976a Pettersson et al 1978) have indicated that splanchnic and vagal nerve stimulation via an adrenergic mechanism causes a release of 5 HT from EC into the portal circulation and possibly also into the lumen (Ahlman et al 1978) The present study strongly suggests that this release of 5 HT is due to a direct neural influence on the EC Luminal stimuli e g acid pH glucose mechanic stimulation (Resnick & Gray 1962 Drapanas et al 1962 Biber 1973) also regulate the release of 5-HT from the EC but the relative importance of ner versus luminal stimuli is not known

This study was supported by grants from the Swedish Medical Research Council (grants no 17X-05224, 04X-2207 04P-4173) The Göteborg Medical Society The Swedish Society of Medical Sciences the Medical Faculty University of Göteborg the M Bergvall's Foundation and H and G Jeansson s Foundation

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mportance of sodium and glucose for the establishment of a villous tissue hyperosmolality by the intestinal countercurrent multiplier

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HALLBÄCK, D A JODAL, M & LUNDGREN O Importance of sodium and glucose for the establishment of villous tissue hyperosmolality by the intestinal countercurrent multiplier Acta Physiol Scand 1979 107 89–96. Received 28 Febr 1979 ISSN 0001-6772. Department of Physiology University of Göteborg, Sweden

The intestinal countercurrent multiplier has earlier been shown to create an increased tissue osmolality in the villi (Jodal et al. 1978). In the present paper the importance of varying the luminal contents on the creation of the villous hyperosmolality was investigated using the cryoscopic technique described by Jodal et al (1978) The perfusion solutions used contained 0, 25 or 147 mmol NaCl and were either provided with mannitol or glucose (30 mmol/l). It was demonstrated that sodium was of particular importance for the establishment of the villous hyperosmolality while glucose only contributed significantly at low luminal sodium concentrations It is therefore proposed that glucose only in the absence of sodium in the luminal perfusate may effectively participate in the generation of the villous tissue hyperosmolality via the countercurrent multiplication mechanism.

It has been proposed by Jodal and coworkers (Haljamäe et al 1973 Jodal 1973 Jodal et al 1978) that the most important physiological implication of the intestinal countercurrent exchanger is its significance for water and sodium absorption by creating a hyperosmolar compartment at the villous tip Such a hyperosmolality has been inferred to be necessary for explaining water absorption in vivo in the absence of or against lumen to plasma osmotic gradient (Curran & McIntosh 1962, Schultz & Curran 1968). The tissue hyperosmolality is accomplished by the xchanger acting as a counter current multiplier According to the original hypothesis of Jodal and coworkers the hyperosmolality may be established in one of two ways. First, the pumping of sodium by the enterocyte into the villous capillaries would increase sodium plasma concentration in the subepithelial capillary network compared to that in the supplying artery Sodium may then diffuse along its concentration gradient from capillary to arterial vessel to be brought back towards the villous tip Second the same end result could be accomplished if the increased sodium concentration produces an osmotic flow of water from the central arterial vessel to the subepithelial capillaries

It was, however pointed out by Haljamäe et al (1973) that the absorption of any solute that increases plasma osmolality (e.g. glucose amino acids) so as to cause a transfer of water from the central artery to the villous capillaries would produce a high tissue osmolality in the way described above for sodium. In the present paper this hypothesis was tested with regard to glucose by determining the villous osmolality when the concentration of sodium and/or glucose was varied in the fluid perfusing the intestinal lumen.

METHODS

A. *Operative procedure*

The experiments were performed on cats anaesthetized I. with chloralose (50 mg/kg b w) after induction with ether The animal had been deprived of food for 24 h and had no obvious signs of intestinal infection. The operative procedures and the recordings of blood flow were similar to those described by Jodal et al. (1975), which should be

able .. Tip and mea osmolality in various portions f intestinal villi ell as total intestinal blood flow uring different xperimental procedures of this study

Osmolality expressed in mOsm/kgH_2O and blood flow as ml/min 100 g tissue weight. *n*-Values after each perfusing olution refer to number of observations for the osmolality results. Mean values ± S.E.

	Tip	From 5 to 30 % of villous length	From 5 to 50 % of villous length	Whole villous	Total blood flow
Jejunum					
Krebs-glucose (30)	1 016±32	690±21	589±17	467±10	46±8 (=9)
Krebs-mannitol (=23)	891±38	645±23	579±18	469_11	31±4 (=9)
Choline-glucose with 25 mmol Na (=15)	737±56	578±36	513±29	429±20	30±3 (9)
Choline-mannitol with 25 mmol Na (22)	647±37	526± 4	480_19	412±11	26+4 (9)
Choline-glucose (11)	658±52	509±35	462±28	401±17	40±5 (9)
Choline-mannitol (=12)	465±36	410±28	391±25	361±18	25_5 (=6)
Choline-maltose (15)	571_29	460_18	425±14	381± 9	30±5 (=6)
Ileum					
Krebs-glucose (36)	954±28	687±19	596±16	481±1	46±8 (8)
Krebs-mannitol (21)	891±40	678±33	596±16	478 14	29±3 (=10)
Choline-glucose ith 25 mmol Na (=14)	776±46	602±29	532± 1	436±11	3_±3 (9)
Choline-mannitol with 25 mmol Na (23)	640±19	543±17	499±15	429±10	26±4 (=9)
Choline-glucose (n 23)	613±19	505±15	461±13	400±8	46±7 (=8)
Choline-mannitol (14)	485±53	413±34	387±26	355±15	7±6 (5)
Choline-maltose (=16)	651±27	545±28	493±26	423±20	32±6 (=6)

RESULTS

The results of this tudy are presented in Tables –4 and in Figs. 1–4. In Table 2 the measurements of tissue osmolality are presented as tip osmolality and as mean osmolality in the upper 5 to 30 5 to 50 and 5 to 100 % of villous length in the various experimental groups. The figures illustrate the osmolality gradient along the villi when exposing them to the different perfusion solutions. The osmolality measurements obtained with the different solutions are compared in Tables 3 (Jejunum) and 4 (Ileum) using Wilcoxon s non-parametric test. Two *P* values are

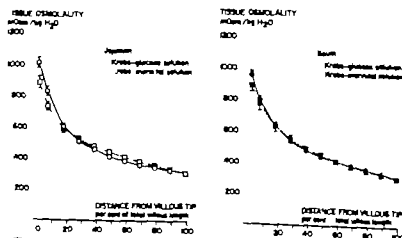


Fig. 1. Tissue osmolality in the villi of jejunum (left panel) and ileum (right panel) when exposed to isotonic Krebs-glucose (circles) and Krebs-mannitol solutions (squares). Bars denote S.E. At many points of the Fig. the S.E. is so small that it falls within the area of the symbols. For number of observations, see Table 2.

Table 1 *Composition of the various solutions of this study*
Concentrations are expressed in mmol/l

	Na+	K+	Mg^{2+}	Ca^{2+}	Choline	Cl	HCO_3	PO_4^{3-}	Glucose	Mannitol	Maltose
Krebs-glucose	147	4 7	1 2	2.5		13[illegible]	25	1	30		
Krebs-mannitol	147	4 7	1 [illegible]	.5		132	[illegible]5	1		30	
Choline-glucose +25 mmol Na	25	4 7	1[illegible]	2.5	122	132	[illegible]5	1[illegible]	30		
Choline-mannitol +25 mmol Na	25	4 7	1 2	[illegible].5	1[illegible]	132	[illegible]5	1.2		30	
Choline-glucose		4 7	1[illegible]	2.5	147	132	[illegible]5	1	30		
Choline-mannitol		4 7	1 2	.5	147	13	[illegible]5	1		30	
Choline-maltose		4 7	1[illegible]	[illegible].5	147	13	[illegible]5	1[illegible]			[illegible]

consulted for details Briefly total venous outflow from two sympathetically denervated intestinal segments (mid jejunum and mid-ileum) was recorded by a drop recorder unit operating an ordinate writer Mean arterial blood pressure was measured from the left femoral artery by a pressure transducer (Statham P23AC) Cholinergic influences were eliminated by i v atropine administration 1 mg/kg b w

B *Recording of intestinal net water transport*

Intestinal net water transport was continuously measured with a recirculating system that was coupled to the lumen of the intestinal segments and contained a reservoir (volume 700–1000 ml) to minimize recirculation The volume change of this system was recorded with a volume transducer connected via a Y-tube to the perfusion system Provided no motility occurred the recorded change of volume reflected net water absorption or secretion (Jodal et al 1975) The intraluminal pressure at the outflow end of the segment was kept at about 1 cmH O and the intestinal segment was perfused at a constant rate of approximately 1 ml/min by means of a roller pump (Model mp-4 Ismatec Sa Zürich Switzerland) The temperature of the perfusate entering the segment was continuously monitored with a thermocouple thermometer (Electrolab Copenhagen) and kept at about 38°C

C *Cryoscopic technique*

1 *General procedure* The cryoscopic method has been presented in detail by Jodal et al (1978) also describing the calibration procedures Briefly after at least 20–25 min of constant net water transport the segments were rapidly extirpated and momentarily frozen in isopentan precooled to −150°C in liquid nitrogen The intestinal wall was then cut obliquely in a cryostat kept at −25°C so as to give cross-sections at different levels of the villi The tissue sections (15 μm thick) were embedded in kerosene between glass plates and transferred to a temperature controlled unit consisting of an aluminium block mounted on a Zeiss standard microscope provided with a camera The temperature of the aluminium block could be set at any temperature between −4.5 and +0.5°C Thawing of the intestinal tissue was achieved by stepwise increases of tissue temperature

In the first part of this study (about one third of the experiments) the temperature of the aluminium block was increased manually and the amount of tissue that was thawed at each temperature level was determined from photographs taken manually at the end of an equilibration period usually lasting about 10 min A Zeiss camera (model C35) adapted to the microscope was used Some of the results obtained with this technique has been reported in an earlier publication (Jodal et al 1978, see Results)

In the latter part of this study the temperature of the intestinal tissue was changed automatically by a device constructed by research engineer Lars Stage The starting temperature the temperature increase per step as well as the number of steps (10 or 16) could be varied The tissue temperature was usually increased in 10 steps with a 5-min equilibration period between each step An automatic Robot Star 50 camera was used in this series.

Determination of tissue osmolality The temperature at which the last ice crystals thawed in a villous cross-section was taken as its melting point The tissue melting point determined in this study was considered to be a measure of the extracellular osmolality The magnification used (about 50 times) was too low to allow any observation of intracellular ice crystals in the villous tissue

The thawing of the intestinal tissue always started at the villous tips and spread gradually downwards until finally the crypt regions the submucosa and the muscularis melted at largely the same temperature The percentage of melted villous tissue for each temperature level was determined from the photographs The data were fed into a computer which calculated the tissue osmolality at 5 10 20 30 40 50 60 70 80 90 % of villous length as well as the mean osmolality between 5 and 30 5 and 50 and 5 and 100 % villous length (Jodal et al 1978)

D *Solutions*

The intestinal mucosa was exposed to seven different solutions The contents of these are given in Table 1 In all expts only one type of solution was perfused through each segment studied

E *Statistics*

Statistical significance was tested using Wilcoxon's non-parametric tests (Siegel 1956)

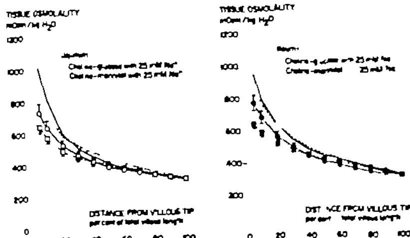


Fig. 2. Tissue osmolality in the villi of jejunum and ileum when exposed to isotonic solutions containing either choline-glucose and 25 mmol Na/l (circles) or choline-mannitol and 25 mmol Na/l (squares). As comparison the tissue osmolality obtained with Krebs-glucose (continuous line) and Krebs-mannitol (dashed line) solutions are indicated in the Fig. For further details consult legend to Fig. 1.

served when the perfusate contained 147 mmol sodium per liter are indicated by thin lines. Comparing the two solutions with 25 mmol sodium did not reach any statistically significant difference between the osmolality values neither at the tip nor between 5 and 30% of villous length (Tables 3 and 4), although the values for the glucose containing solution always were higher.

Fig. 3 illustrates the osmotic gradients along the villus measured when the intestinal perfusate contained no sodium. Data obtained with perfusates containing glucose or mannitol are presented both for jejunum and ileum in a manner similar to that seen in Figs. 1 and 2. As a comparison the osmotic gradients obtained when the perfusing solutions contained 147 (Fig. 1) or 25 mmol sodium per liter (Fig.) are indicated by thin lines. The difference between the tip osmolalities observed with the two sodium free solutions were statistically significant (Tables 3 and 4), the glucose experiments exhibiting

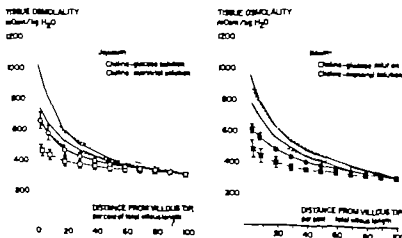


Fig. 3. Tissue osmolality in the villi of jejunum and ileum exposed to choline-glucose (circles) or choline-mannitol (squares) solutions, illustrated in the same way as in Fig. 1. For comparison the tissue osmolalities illustrated in Figs. 1 and 2 are also indicated in the Fig.

Table 3 *P values when comparing villous tip osmolality (upper value) and mean tissue osmolality in the upper 5 to 30 % of villous length (lower value) in the different experimental series performed on the jejunum* The statistical significance was tested using the nonparametric method of Wilcoxon ns = not significant

	Krebs mannitol	Choline-glucose + 25 mmol Na	Choline-mannitol + 25 mmol Na	Choline glucose	Choline-mannitol	Choline-maltose
Krebs-glucose	<0.01 ns	<0.01 <0.01	<0.01 <0.01	<0.01 <0.01	<0.01 <0.01	<0.01 <0.01
Krebs-mannitol		<0.02 <0.05	<0.01 <0.01	<0.01 <0.01	<0.01 <0.01	<0.01 <0.01
Choline-glucose +25 mmol Na			ns ns	ns ns	<0.01 <0.01	<0.02 <0.01
Choline-mannitol +25 mmol Na				ns ns	<0.01 <0.01	ns ns
Choline-glucose					<0.05 ns	<0.05 ns
Choline-mannitol						ns ns

given for each comparison the upper one was obtained when testing the tip osmolality values and the lower one when comparing mean osmolality values in the upper 5 to 30 % of the villi

Fig 1 illustrates the compiled results of measuring the osmotic gradients along the villus when the perfusing solutions contained either glucose (continuous line) or mannitol (dashed line) and the sodium concentration was 147 mmol/l Data are presented both for the jejunum (left panel) and the illeum (right panel) In the jejunum the tip osmolality when using glucose solution was significantly higher than when perfusing with mannitol solution but not so in the ileum (Tables 3 and 4) The tip osmolality of the 4 experimental groups presented in Fig. 1 are somewhat lower than similar values presented in an earlier report (Jodal et al 1978) which may be attributed to the modified cryoscopic technique (see Methods)

Fig. 2 illustrates in the same way as in Fig. 1 the osmotic gradients along the villus when the sodium concentration in the perfusing solutions was 25 mmol/l and the perfusates contained either glucose or mannitol The osmolality of the perfusates was maintained around 317 mOsm/kgH_2O by addition of choline chloride (Table 1) The results obtained with the perfusate containing glucose (continuous line) or mannitol (dashed line) are presented both for the jenunum (left panel) and the ileum (right panel) For comparison the osmotic gradients ob-

Table 4 *P Values when comparing villous tip osmolality (upper value) and mean tissue osmolality in the upper 5 to 30 % of villous length (lower value) in the different experimental series performed on the ileum* The statistical significance was tested using the nonparametric method of Wilcoxon ns = not significant

	Krebs mannitol	Choline-glucose + 25 mmol Na	Choline-mannitol + 25 mmol Na	Choline glucose	Choline mannitol	Choline-maltose
Krebs-glucose	ns ns	<0.01 <0.05	<0.01 <0.01	<0.01 <0.01	<0.01 <0.01	<0.01 <0.01
Krebs-mannitol		<0.05 ns	<0.01 <0.05	<0.01 <0.01	<0.01 <0.01	<0.01 <0.01
Choline-glucose +25 mmol Na			ns ns	ns ns	<0.01 <0.01	ns ns
Choline-mannitol +25 mmol Na				ns ns	<0.01 <0.01	ns ns
Choline-glucose					<0.01 <0.01	ns ns
Choline-mannitol						<0.01 <0.01

different in two cases. In the jejunum a statistically significant difference could be demonstrated between maltose and glucose and in the ileum between maltose and mannitol.

Total blood flow in the two intestinal segments including their lymph nodes are shown in Table 2. The average blood flow in segments perfused with a solution containing glucose was consistently higher than in the corresponding groups with mannitol perfusate. No statistically significant difference could however be demonstrated between individual groups. Comparing all flow values recorded in experiments using a glucose containing perfusate with flow rates in all mannitol groups revealed a highly significant difference ($P<0.01$).

DISCUSSION

The effect of glucose on villous tissue hyperosmolality was investigated by comparing the experimental results recorded when segments of jejunum and ileum were perfused with isotonic solutions containing either glucose or mannitol. Furthermore to evaluate the sodium and glucose interdependence experiments were performed at 3 different sodium concentrations, i.e. 0, 25 and 147 mmol/l (Figs. 1–3). Osmolality in the different solutions was kept constant by addition of choline (Table 1). When the sodium concentration in the perfusing solution was high (147 mmol/l) addition of glucose induced only a small rise in villous tissue osmolality compared with the mannitol solution (Fig. 1, Tables 2 and 3). The increase of tissue osmolality produced by the addition of glucose was, however, larger with diminishing sodium concentration, and thus the largest raise in tissue osmolality was observed when sodium concentration in the perfusing medium was zero (Tables 2 and 3). An increase of tissue osmolality with increasing sodium concentration in the intestinal perfusate was also demonstrated (Figs. 1–3 and Table 2).

The result of the present study hence suggest that glucose may contribute to the establishment of an osmolar gradient in the villus particularly in the absence of sodium in the perfusate. This hypothesis infers that the absorbed glucose increases the blood glucose concentration in the villous capillaries (see Introduction). This can be accomplished in two ways. In vitro studies have provided evidence for a facilitated diffusion process at the baso-lateral membranes of the epithelium (Murer & Hopfer 1977). This process implies among other things the presence of an intracellular glucose concentration that is higher than that of plasma. In vivo, however, no such intracellular accumulation of glucose has been shown, indicating the possible presence of an active transport system (Esposito et al. 1977). Both mechanisms are probably able to create the necessary increase in plasma glucose concentration mentioned above.

The insignificant effect on tissue osmolality of glucose at high sodium concentrations in the perfusate may be explained by a highly efficient and fast active transport of sodium into the intestinal capillaries, making the transport of glucose quantitatively unimportant. Moreover, the slight hyperemia in the glucose experiments as compared to the mannitol ones might have decreased villous osmolality by e.g. reducing the efficiency of the countercurrent multiplier (cf. Jodal et al. 1978). The results with glucose only partly corroborate the hypothesis presented by Fordtran and coworkers (Fordtran et al. 1968, Fordtran 1975), which implies that glucose is the main solute determining net water absorption. However, at low or zero sodium concentration in the luminal solution this hypothesis is in better agreement with the results of the present study.

In this context it should be pointed out that the sodium concentration close to the villous surface is probably not zero under in vivo conditions, even if the entering perfusing solution is devoid of sodium. This is for two reasons. First, there is a continuous sodium diffusion from tissue to lumen along a favorable concentration gradient; second, the sparse sodium ions may have an uneven distribution in the lumen due to a negative charge of the glycocalyx which enhances sodium concentration close to the villous surface. Therefore one cannot entirely exclude the possibility that the effect of glucose on tissue osmolality may in the sodium free perfusate be induced by enhancing active sodium transport at the brush border (Crane 1962, Schultz & Curran 1968, 1970).

In an attempt to test this possibility the intestinal segments were perfused with solutions containing maltose instead of glucose. These experiments were based on the observation by Crane and coworkers (for ref. see Caspary 1977) that glucose molecules produced by a splitting of maltose are absorbed via a sodium independent mechanism into the enterocyte. This mechanism has, however, been considered to be of little quantitative impor-

Table 5 *Increase in villous osmolality in the upper 5 to 30% of villous length produced by the addition glucose to the perfusate*

The increase is expressed in mOsm/kgH_2O and in % of the osmolality values observed when perfusing the intestinal lumen with a solution containing no glucose

Concentration of sodium in perfusate (mmol/l)	Jejunum mOsm/ kgH_2O	Jejunum %	Ileum mOsm/ kgH_2O	Ileum %
147	45	7	9	1
25	52	10	59	11
0	99	24	92	22

a higher osmolality A significantly higher mean osmolality in the upper 30% of villous length could only be demonstrated in the ileum

The results given in Fig. 3 clearly demonstrate the importance of increasing sodium concentration in the solution perfusing the intestinal lumen particularly in solutions devoid of glucose Stepwise increases of the sodium content in the mannitol solutions from zero to 147 mmol/l significantly increased villous osmolality (Tables 3 and 4) In the experiments with glucose containing solutions the increase of sodium concentration from 25 to 147 mmol/l produced a statistically significant osmolality increase while the rise from zero to 25 mmol/l induced no significant effect (Tables 3 and 4)

Table 5 illustrates in another way the effect of glucose on tissue osmolality in solutions with varying sodium concentrations This Table is based on the average values given in Table 2 The observed increase in tissue osmolality in the upper 30% of villous length upon addition of glucose to the perfusate is most pronounced in the solutions devoid of sodium Similar results were obtained when expressing the osmolality rise in per cent of the value observed when exposing the tissue to a glucose free solution

In one series of expts the intestinal mucosa was exposed to a sodium free solution containing the disaccharide maltose The observed villous osmolality gradient is illustrated in Fig. 4 As a comparison the osmotic gradients recorded when the perfusate contained glucose or mannitol but no sodium are also shown Comparing the tip osmolality obtained with the maltose solution with those seen with the two sodium free solutions containing glucose or mannitol showed that they were statistical

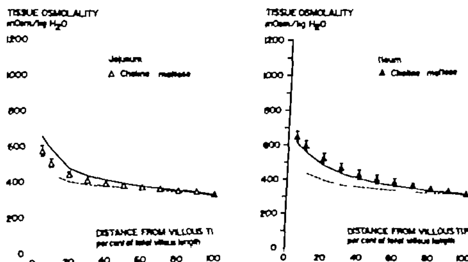


Fig 4 Tissue osmolality in the villi of jejunum and ileum when exposed to a choline maltose solution (triangles) For comparison the tissue osmolalities obtained with choline-glucose (continuous line) and choline-mannitol (d hed line) solutions are indicated in the Fig

Release of prostaglandin-like substances during elevations of left atrial pressure in the cat

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WIBERG, T VAAGE, J & SCOTT E. Release of postaglandin-like substances during elevations of left atrial pressure in the cat. Acta Physiol Scand 1979 107 97–103. Received 31 Jan 1979 ISSN 0001-6772. Institute of Physiology University of Oslo, Norway

The purpose of the present ork was to measure prostaglandin (PG)-lik activity in arterial blood during acute pulmonary hypertension Anesthetized cats with the chest opened and given positive pressure ventilation were used A balloon in the left atrium was inflated to elevate hydrostatic vascular pressure in the lungs. Blood was pumped (10 ml/min) from carotid artery to superfuse 3 smooth muscle tissues rat stomach strip, rat colon and chick rectum, the blood was then returned to the jugular vein by gravity The assay tissues were pretreated ith antagonists against catecholamines, histamine, serotonin and acetylcholine during the experiments They were sensitive to calibrating doses of 2 ng/ml of PGF$_{2\alpha}$ and 1 ng/ml of PGE$_2$. 18 periods with elevated left atrial pressure (P_{LA}) (?1–49 mmHg), lasting 26 min, were applied in 9 cats This manoevre usually also caused systemic hypotension 14 of these P_{LA} elevations were accompanied by increased arterial PG-like activity which rapidly subsided when the pressure was released or when indomethacin (? mg/kg =4) was given In 3 additional experiments it was found that pulmonary degradation of PGs was unaffected during P_{LA} elevation I Infusion of angiotensin II contracted the tissues in a pattern different from that caused by pressure elevations and the PG calibrations, and these contractions were not affected by indomethacin. This indicates that the assay tissue contractions cannot be caused by angiotensin II which alone does not increase PG-like activity in arterial blood Consequently acute pulmonary ascular hypertension appears to stimulate PG synthesis and release in lungs of intact cats

Key ords cats, lung, pulmonary hypertension; prostaglandins, angiotensin superfusion method

The lungs ha e large capacity for PG synthesis hich is stimulated by various mechanical stimuli such as squeezing, stroking or stirring (Piper & Vane 1971 Bakhle & Vane 1974) Distortion of cell membranes has been suggested to be a common denominator in stimulation of PG ynthesis and re lease (Piper & Vane 1971) Stretching of the lungs by increasing airw y pressure has been suggested to release PGs (Berry Edmonds & Wyllie 1971 Said 1974 Vaage Wiberg & Scott 1978) Pulmonary congestion and hypertension which lead to stretching of essel walls and interstitial lung tissue may also stimulate PG ynthesis. Pulmonary ynthesis and release of PGs may influence pulmonary func tion (Mathe et al 1977) and PGs released int the blood stream may ha e profound effect on the ystemic circulation as well (Malik & McGiff 1976)

We wanted to investigate whether elevation of hydrostatic pressure in the pulmonary circulation caused by elevation of left arterial pressure induced release of prostaglandin-like substances (PGLS) in the intact animal.

Additionally we ha e tested if increased amounts of PGLS were due to reduced pulmonary inactivation of PGs and whether PGLS were ynthetized secondary to release of angiotensin II Some of our findings have been presented in a preliminary communication (Wiberg et al 1976)

METHODS

Cats weighing 3.3–4 3 kg were used for the experimental procedure cats weighing 1.8–.3 kg were used as blood donors. All cats were anesthetized with pentobarbitone sodium, 30–40 mg/kg, given i p. The blood donors were

tance (Caspary 1977 Semenza 1977) and the present experiments also failed to show any conclusive effect on tissue osmolality of the disaccharide

To summarize the present study has demonstrated that the creation of a villous hyperosmolality is dependent on the contents of the solution perfusing the intestinal lumen It has been shown that the presence of sodium is of particular importance in this respect while glucose may be contributing at low luminal sodium concentrations

When comparing blood flow in intestinal segments perfused with isotonic solutions with glucose or mannitol it was noted that the intestinal blood flow was significantly higher in the animals perfused with a glucose solution It seems reasonable to propose that this vasodilatation represents a functional hyperemia produced by the luminal contents Two underlying mechanisms seem possible the release of hormones (Fara et al 1972) and/or the activation of local nervous reflex(es) (Biber 1973 Post et al 1975)

This research was supported by grants from the Swedish Medical Research Council (14X 2855) from the Swedish Society for Medical Sciences from Harald and Greta Jeanssons Fund from Magnus Bergvalls Stiftelse and from the Faculty of Medicine University of Göteborg.

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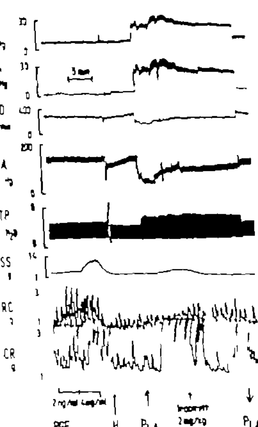


Fig. 1. The effects of levating left atrial pressure in an anaesthetized cat with the chest opened and ventilated with constant tidal volume. The tracings are from the top: Pulmonary arterial pressure (P_{PA}), left atrial pressure (P_{LA}), cardiac output (CO), femoral arterial pressure (P_A), transpulmonary pressure (P_{TP}) and isometric tension in 3 bioassay tissues continuously superfused with arterial blood (10 ml/min): rat stomach strip (RSS), rat colon (RC), and chick rectum (CR). The assay tissues are calibrated by direct administration of $PGF_{2\alpha}$. H indicates hyperinflation of the lungs. During P_{LA} elevation indomethacin was given.

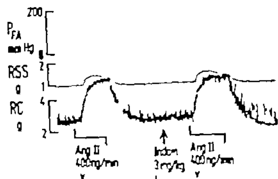


Fig. 2. Effect of infusing angiotensin II L to an anesthetized cat (cardiac output 400 ml/min) on femoral blood pressure (P_A) and 2 bioassay tissues continuously superfused by arterial blood (10 ml/min): rat stomach strip (RSS) and rat colon (RC) before and after i administration of indomethacin to block PG synthesis.

formed hen baseline tension of the assay tissues had stabilized in blood and reacted to standard doses of 1 and 2 ng/ml of PGE_2 and 2 and 4 ng/ml of $PGF_{2\alpha}$. T counteract the tendency towards spontaneous airways collapse and microatelectasis the lungs were inflated to peak P_{TP} of 70 cm H_2O for few seconds 5 min before elevation of P. The left atrial balloon as inflated to give P of 30–40 mmHg. I some cases the highest P hich the animals could endure with stable although lowered, ystemic blood pressure and cardiac output, was employed. I most animals consecutiv pressure elevations ere performed w h intervals of at least 30 m . PG calibrations ere performed between each test.

T investigate hether pulmonary hypertension and congestion influenced pulmonary inactivation of PGs, cat were prepared as described but they were given indomethacin (2 mg/kg i.) before tests. In the control situation an infusion of PGE_2 was given at dose hich contracted the assay tissues corresponding to about 2 ng/ml. When the ssay tissues had reached plateau P_{LA} was increased with the infusion of PGE_2 going, to test if change in PGE_2 concentration in arterial blood occurred in relation to pressure elevation.

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P lmonary nd cardiovascular effect of ed P_{LA}

When the balloon in the left atrium was inflated P_{LA} and P increased and remained elevated until the balloon was deflated. Simultaneously P_{TP} increased moderately whereas cardiac output P and pulmonary vascular resistance decreased. These changes in P_{LA} and P are presented in Table 1. Pulmonary and cardiovascular changes in the same model have previously been described in more detail (Hauge, Bø & Aarseth 1977).

Release of PGLS

A total of 18 P elevations were performed in 9 cat (Table 1). During 14 of these elevations the ssay tissues contracted suggesting release of PGLS within a couple of min (Fig. 1). The contractions were maintained but subsided rapidly when

Table 1. *Effect of increased P_{LA} on PG release*

Cat no.	Pressure elevation no.	P_{LA} (mmHg)	Duration of P_{LA} elevation (min)	PG-like substances (estimated as PGE_2)	P_A before and during P_{LA} elevation (mmHg)	Indomethacin infused
1	1	4	20	0	130–120	
1		4[illegible]	6	0	130–120	
1	3	43	1	4	110–100	
1	4	[illegible]5		1	80–70	
1	5	[illegible]1	7	PGLS	70–65	
	1	30	26	1	110–85	
		26	1	0	110–85	
3	1	3[illegible]	11	4	90–60	
4	1	30	10		140–85	
4		[illegible]	20	3	135–[illegible]5	+
5	1	33	11		1[illegible]5–80	
5		30	4	[illegible]	95–[illegible]5	+
6	1	30	16	PGLS	115–80	
	1	49	15		115–90	
7		40	13	0	90–65	
	3	30	10	PGLS	85–70	
8	1	30	1		90–70	
9	1	31	1	PGLS	90–70	+

PGLS indicates that type of PG could not be specified.

heparinized with 500 I.U./kg and exsanguinated by cardiac puncture. To each 100 ml of this blood was added 1000 I.U. dry power heparin. The surgical procedures have previously been described in detail (Vaage, Bø & Hognestad 1974).

Ventilation. A tracheal cannula was inserted, a muscle relaxant (Alloferine, Hoffmann-La Roche, 0.5 mg/kg) was given, and positive pressure ventilation with constant tidal volume was started. End-expiratory pressure was kept at +1.5 cm H_2O by means of a water seal. The respiratory frequency was [illegible]/min and the tidal volume was adjusted so as to keep pH in arterial blood between 7.35–7.45 at the onset of each experiment and was then kept at this level throughout the expt. Tracheal pressure, which in this model equals transpulmonary pressure ($P_{[illegible]}$), was recorded by a Statham P 23 Dc transducer via a side-arm of the tracheal cannula.

Vascular pressures, left atrial pressure and flow recordings. The thorax was opened widely by splitting the sternum. Polyethylene catheters were introduced into the femoral artery and into the pulmonary artery through the right ventricular wall for recording of femoral arterial pressure (P_A) and pulmonary arterial pressure (P_{PA}) respectively. A balloon catheter (U.S. Cates No. 8) was placed into the left atrium to record left atrial pressure (P_{LA}). P_{LA} could be elevated by inflating the balloon. All pressures were monitored by Statham transducers connected to a Grass polygraph (Model 7B, Grass Instrument CO., Quincy, Mass., USA). Cardiac output (minus coronary flow) was measured by an electromagnetic flow probe placed around the ascending aorta. Both pulsatile and mean flow were recorded by a Nycotron square wave flowmeter (type [illegible]) Nycotron, [illegible] Norway) connected to the Grass polygraph.

Bioassay. A modification of the blood bathed organ technique (Vane 1969) was used as described by Vaage, Wiberg & Scott (1978). When the surgical procedure was completed the cat were given 1000 I.U./kg of heparin i.v. From a catheter in the left carotid artery 10 ml/min of blood was pumped to superfuse a series of assay tissues: rat stomach strip (RSS), rat colon (RC) and chick rectum (CR). The blood then returned to a jugular vein by gravity. The tissues were pretreated with blockers against catecholamines, histamine, serotonin and acetylcholine (Vaage, Wiberg & Scott 1978). In 4 expts spirally cut strips of rabbit aorta (RbA) were included as well. Isometric tension of the assay tissues were recorded by semiconductor transducer elements connected to the Grass polygraph. Upstream to the assay tissues standard doses of PGE_2, $PGF_{2\alpha}$ or angiotensin II were infused for calibration.

Transfusion. When the extracorporeal circulation was started, cat whole blood was infused to compensate for the blood lost into the extracorporeal circuit (20–25 ml). Cat blood alone, isotonic saline or dextran (Macrodex, Pharmacia, Sweden) was given throughout the expts whenever necessary to maintain blood pressure and cardiac output.

Inhibition of PG synthesis. Indomethacin dissolved in a phosphate buffer (5 mg/ml) was given [illegible] mg/kg to inhibit PG synthesis (Vane 1971).

pH measurements in arterial blood were carried out with an Acid-Base Analyzer PHM 71 Radiometer, Copenhagen, Denmark.

Hematocrit was determined in duplicate by a microhematocrit centrifuge.

Osmolality was determined in plasma with an Advanced Digimatic Osmometer (Advanced Instruments Inc., Needham Heights, Mass., USA).

Experimental protocol. Pressure elevations were per-

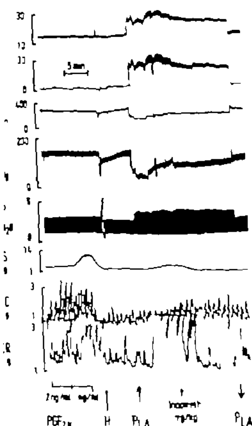


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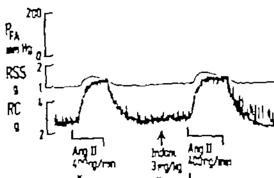


Fig 2 Effects of infusing angiotensin II L to an anesthetized cat (cardiac output 400 ml/min) on femoral blood pressure (P) and 2 bioassay tissues continuously superfused by arterial blood (10 ml/min), rat stomach strip (RSS) and rat colon (RC) before and after L administration of indomethacin to block PG synthesis

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1	4	25		1	80– 70	
1	5	1	7	PGLS	70– 65	
2	1	29	26	1	110– 85	
	2	26	21	0	110– 85	
3	1	3	11	4	90– 60	
4	1	30	10	2	140– 85	
4		28	20	3	135– 75	+
5	1	33	11		1_5– 80	
5	2	30	4		95– 75	+
6	1	30	16	PGLS	115– 80	
7	1	49	15	2	115– 80	
7	2	40	13	0	90– 65	
7	3	30	10	PGLS	85– 70	
8	1	30	1		90– 70	+
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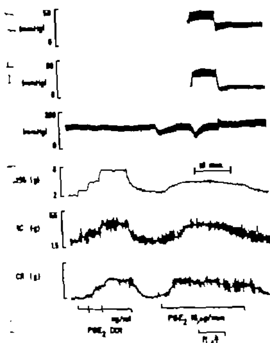


Fig. 4. Effect of P elevation on pulmonary degradation of infused PGE_2. The tracings are pulmonary arterial pressure (P_{PA}), left atrial pressure (P_{LA}), femoral arterial pressure (P_A), and isometric tension in 3 bioassay tissues continuously superfused with arterial blood (10 ml/min): rat stomach strip (*RSS*), rat colon (*RC*) and chick rectum (*CR*). Indomethacin 2 mg/kg was given before the start of the tracings. The tissues were calibrated by PGE_2 and the lungs hyperinflated (*H*). P_{LA} was elevated during an infusion of PGE_2.

Pulmonary hypertension and inactivation of PGE

This was investigated in 3 cats given indomethacin to prevent PG synthesis. After calibration of the assay tissues PGE_2 was infused at a dose which caused assay tissues contractions corresponding to about ng/ml in arterial blood. Inactivation was found to be between 81 and 97 % and was not influenced by elevation of P_{LA} (Table 2) (Fig. 4).

Hematocrit (hct) and osmolality

At the onset of each expt. hct was 27–35 (range) and declined to 15–28 (range) throughout the expt., probably due to hemodilution caused by transfusions.

Plasma osmolality was unchanged at 370–328 mosmol/l throughout each expt. It did not change during pressure elevations.

DISCUSSION

In the present work we have focused on increased hydrostatic pressure in the pulmonary circulation as a possible stimulus of PG synthesis and release from intact cat lungs. Inflation of a balloon in the left atrium causes pulmonary hypertension and congestion as well as systemic hypotension (Hauge Bo & Aarseth 1977). Simultaneously the bioassay tissues contracted indicating release of PGLS. The presently used bioassay tissues are specifically sensitive to PGs (Piper & Vane 1971). Counteracting their contractions by administration of indomethacin, an inhibitor of PG synthesis, is further proof that PGLS really were released since PG release represents de novo synthesis (Piper & Vane 1971).

The assay tissues were calibrated by known concentrations of the "classical" PGE_2 and $PGF_{2\alpha}$. Due to the different contraction pattern of the 3 tissues, the PGLS released could be classified as PGE_2 or $PGF_{2\alpha}$-like material. However, during the conversion of arachidonic acid (AA) in lungs only a minor fraction is transferred into PGE_2 and $PGF_{2\alpha}$. A major fraction of the endoperoxide intermediates (PGG_2 and PGH) is converted to the highly unstable, but biologically very active thromboxane A (TxA_2) (Hamberg, Svensson & Samuelsson 1975). Conceivably endoperoxides and TxA_2 are released in far higher concentrations than PGE_2 and $PGF_{2\alpha}$ in the present expts. Our assay tissues are also sensitive to TxA_2 and endoperoxides (Hamberg et al. 1975, Bunting, Moncada & Vane 1976). The use of the RbA to detect RCS, which is a mixture of TxA_2 and endoperoxides (Hamberg, Svensson & Samuelsson 1975, Samuelsson 1976) confirmed the release of these intermediates. Recent investigations have indicated that AA in the lungs is also transformed into the vasodilatory PGI (Dusting et al. 1978). PGI is also able to contract the RSS and the CR (Omini, Moncada & Vane 1977). Consequently the presently used on-line bioassay may reflect the biological activity and variety of substances formed during the biological conversion of AA.

In the present expts. we could not detect any release of PGLS in 4 of the 18 elevations of P_{LA}. This might indicate that release is not a constant phenomenon, or that in some expts. the amounts released were too small to be detected. The release amounts which w detected, are close to the lower level possible to detect by bioassay. Release of

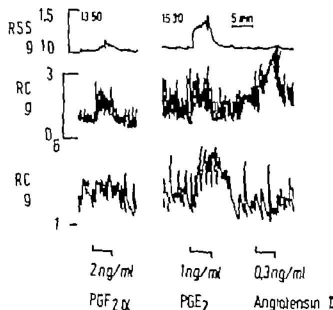


Fig 3 Typical effects of standard doses PGE PGF$_{2\alpha}$ and angiotensin II on 3 bioassay tissues superfused with arterial blood (10 ml/min) from an anesthetized cat The assay tissues are from the top rat stomach strip (*RSS*) rat colon (*RC*) and chick rectum (*CR*)

P_{LA} was returned to normal When compared to the pattern of tissue contractions obtained by standard doses of PGE$_2$ and PGF$_{2\alpha}$ PGF$_{2\alpha}$ like material (1–4 ng/ml) was released in 10 cases In the remaining 4 the contractions could not be matched by the cali brations The tissues usually contracted to 1 ng/ml of PGE$_2$ and 1–2 ng/ml of PGF$_{2\alpha}$ During control periods (P_{LA} 1–5 mmHg) no definite change in PG like activity was observed

During 4 elevations of P_{LA} no release of PGLS was detected The experimental conditions in these 4 situations in 3 cats were not different from the rest of the group (Table 1) In all these cats however release of PGLS occurred during other pressure elevations (Table 1)

During ? pressure elevations gross alveolar ede ma developed judged from foam pouring out of the trachea and P_{TP} increased throughout pressure e vation and did not normalize afterwards (Hauge, I & Aarseth 1977) Only one of these cases was a companied by release of PG like activity (Table 1)

In cats 6 to 9 a rabbit aorta (RbA) was used In of the 6 pressure elevations PGLS were detected in all four tissues

Effect of indomethacin

During 4 elevations of P_{LA} accompanied by defin contractions of the assay tissues indomethacin (mg/kg) was given i v to inhibit PG-synthesis In al these cases the tension of the tissues returned to the control level within a few min (Fig 1). No furthe change in ten ion was observed when P_{LA} later re turned to normal after deflating the balloon

Possible interference of angiotensin II

In all cats a reduction in P_{LA} occurred when P was elevated (Table 1) Sometimes a grav hypotension developed This could activate the re nin angiotensin system with generation of an giotensin II which might interfere with our assa since the tissues were not blocked against an giotensin II To evaluate this possibility angiotensin II was infused 6 times in 4 cats both i v and directl over the assay tissues to give a blood concentration of 0 1–1 0 ng/ml The tissue reactions to angiotensin II were independent of the route of administration In 2 cats angiotensin II was infused twice i v Be fore the second infusion indomethacin (3 mg/kg) was given However the tissue contractions before and after indomethacin were identical (Fig 3) indi cating that this blood level of angiotensin II does not increase PG-like activity in arterial blood Addi tionally the pattern of tissue contractions to an giotensin II was dissimilar to that of PGE and PGF$_{2\alpha}$ Angiotensin II especially contracted the RC powerfully (Fig 3)

Table 2 *Effect of increased P_{LA} on pulmonary degradation of PGE*

Cat no	Cardiac output (ml/min)	PGE$_1$ infusion i v (μg/min)	Activity in art blood (ng/ml)	P (mmHg)	Activity in arterial blood during pressure elevation (ng/ml)	% degradation
		15		5	2	9
10	600		3	30	3	81
11	650	10		77	2	88
1	900	15				—

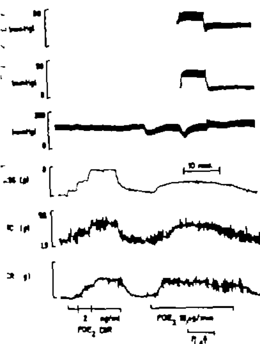


Fig. 4. Effect of P_{LA} elevation on pulmonary degradation of infused PGE$_2$. The tracings are pulmonary arterial pressure (P_{PA}), left atrial pressure (P_{LA}), femoral arterial pressure (P) and isometric tension in 3 bioassay tissues continuously superfused with arterial blood (10 ml/min): rat stomach strip (*RSS*), rat colon (*RC*) and chick rectum (*CR*). Indomethacin 2 mg/kg was given before the start of the tracings. The tissues were calibrated by PGE$_2$ and the lungs hyperinflated (*H*). P_{LA} was elevated during infusion of PGE$_2$

Pulmonary hypertension and inactivation of PGE$_2$

This was investigated in 3 cats given indomethacin to prevent PG synthesis. After calibration of the assay tissues PGE$_2$ was infused at a dose which caused assay tissues contractions corresponding to about ng/ml in arterial blood. Inactivation was found to be between 81 and 92% and was not influenced by elevation of P_{LA} (Table 2) (Fig. 4)

Hematocrit (hct) and osmolality

At the onset of each expt. hct was 27–35 (range) and declined to 15–28 (range) throughout the expt., probably due to hemodilution caused by transfusions.

Plasma osmolality was unchanged at 320–328 mosmol/l throughout each expt. It did not change during pressure elevations.

DISCUSSION

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smaller amounts of PGLS would have escaped detection

The systemic hypotension occurring during elevation of P_{LA} might cause generation of angiotensin II which again might contract the assay tissues Indeed the contraction of the RC during P_{LA} elevation could probably be completely explained by the effect of angiotensin II However angiotensin II can neither explain the contractions of the RSS and the RC nor the effect of indomethacin But angiotensin II might be released together with PGLS What we have characterized as $PGF_{2\alpha}$ like substances might be PGE_2 like substances plus angiotensin II Angiotensin II itself is also reported to stimulate PG synthesis and release in several tissues and cells (McGiff et al 1970 Gimbrone & Alexander 1975) However in the present expts it appears unlikely that PGLS were released secondary to release of angiotensin II I v infusions of angiotensin II did not increase PGLS in arterial blood The increase in blood level of angiotensin II during our infusion was higher than the maximal blood concentrations (0 33 ng/ml) described during hypovolemia (Hodge et al 1966)

A main question is the site of origin of the PGs If they are released from extrapulmonary tissues then they would have to pass the lungs to appear in arterial blood Since the catabolism and inactivation of PGs is extremely efficient in the pulmonary circulation (70–90%) (Ferreira & Vane 1967 Piper Vane &Wyllie 1970) this situation would need extremely high levels of PGs in the mixed venous blood reaching the lungs unless PG degradation was impaired If the pressure elevation significantly increased shunting of blood outside the pulmonary microvasculature during pressure elevation PGs originating in the systemic circulation might escape degradation and thereby increase systemic arterial concentrations of PGs We tested the degradation of PGE_2 in our model (Table) and could show that the pulmonary inactivation of PGE_2 (81–92%) was not changed by elevation of P_{LA} The values we found for degradation were close to the values given by Ferreira & Vane (1967) in cat Consequently if the PGLS released do not originate in the lungs then mixed venous blood must have a concentration of PGs higher than 10 ng/ml to cause the activity on the assay tissues Thus it appears highly unlikely that the PGLS released have an extrapulmonary site of origin

The mechanical stress due to increased hydrostatic vascular pressure in the pulmonary circulation and extravascular accumulation of fluid might theoretically stimulate PG synthesis Said & Yoshida (1974) reported that lung edema stimulated PG synthesis and release from lungs However in the presently used model fluid accumulation in the interstitium is usually scarce unless P_{LA} is excessively high (Hauge Bo and Aarseth 1977) In one of the two cases where fulminant alveolar edema developed no PGLS appeared Recently we have found in isolated perfused lungs that no PGLS were detected in spite of gross alveolar edema and hypertension (Scott Vaage & Wiberg 1979), indicating that the release detected in the present work is dependent on the intact state of the animal and probably not due to direct mechanical effect on the lungs

The elevation of P_{LA} is a marked strain on the animal It must inevitably be accompanied with increased activity in the sympathetic nervous system and release of catecholamines which again might cause PG release (Hedqvist 1976) Interestingly stimulation of sympathetic nerves to the lungs has been reported to release PGs (Mathé et al 1977) Release of PGs from lungs secondary to increased activity in the sympathetic nervous system may explain the difference between the findings in intact animals and in isolated lungs during elevated vascular pressure in the pulmonary circulation

T Wiberg and J Vaage were research fellows of the University of Oslo F Scott was a research fellow of the Ciba-Geigy Fellowship Trust London

This work was supported by grants from the Norwegian Council on Cardiovascular Disease the Norwegian Research Council for Science and the Humanities and Norsk Medisinaldepot PGs were the generous gift of Dr J E Pike Upjohn Company

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Effects of acute hypotension and hypertension on serum TSH concentrations in male rats

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MANNISTO P T KOKKONEN J & RANTA T Effects of acute hypotension and hypertension on serum TSH concentrations in male rats. Acta Physiol Scand 1979 107 105–107 Received 18 Febr 1979 ISSN 0001-6772. Department of Pharmacology University of Helsinki Department of Pharmacology University of Kuopio and Department of Serology and Bacteriology University of Helsinki Finland.

The effect of acute hypertension and hypotension on serum TSH concentration was studied in anesthetized male rats. I v infusions (10 and 30 min) of Na-nitroprusside and dihydralazine induced profound hypotension, and angiotensin amide and noradrenaline increased blood pressure but none of the treatments significantly modified serum TSH concentrations Also clonidine and noradrenaline when given i p caused hypertension, but again the increase of serum TSH levels was not consistent When the whole material was analysed, there was scarcely significant correlation between the change of blood pressure and the change of serum TSH level It is inferred that the drugs affecting TSH secretion, do not exert their action solely by changing the blood pressure

Key words hypertension, hypotension serum TSH noradrenaline, clonidine dihydralazine sodium nitroprusside angiotensin amide

When studying the neurotransmitter control of thyrotrophin (TSH) secretion we (Tuomisto et al 1975 Ranta et al 1977 Mannisto & Ranta 1978) and others (Onaya & Hashizume 1976 Annunziato et al 1977 Krulich et al 1977) have used drugs which besides affecting tissue biogenic amines, alter the blood pressure of the animals There is only a limited information about the effect of some antihypertensive drugs on the brain blood-flow Na-nitroprusside does not significantly affect brain perfusion (Griffiths et al 1974) but clonidine may initially decrease the brain blood flow both in man (James et al 1970) and in animal (Sherman et al 1968) Concerning the anterior pituitary Porter & coworkers (1967) have shown that the blood flow in the pars distalis is markedly increased during a profound hypotension Hence it is possible that rapid change of the blood pressure may alter the blood flow in the pituitary portal vessels, and may so modify the secretion of anterior pituitary hormones. To explore this possibility we have performed a series of experiments, where the blood pressure cardiac rate and serum TSH levels were monitored while various drugs, inducing either hypotension or hypertension were continuously infused We also used drugs whose primary action is known not to be mediated through the brain biogenic amines

MATERIAL AND METHODS

Male Wistar albino rats (*Rattus norvegicus*), weighing 200–290 g were used at the age of 7–8 weeks. The animals were fed pelleted rat chow (iodine content about 0.8 mg/kg; Hankkija, Finland) and tap water ad lib. They were kept in groups of 4–5 in dark room, artificially illuminated from 7 a m. to 9 p m. at a temperature of 22 24°C

The rats were anesthetized with pentobarbital (50 mg/kg, i p.) Usually 2 3 rats were operated on in one experiment. Arteria carotis (usually left) and Vena jugularis (usually right) were cannulated. The cannula in A. carotis was connected to a pressure transducer (Nihon Kohden TMI MPU-0 5-290-0-III), to an amplifier and a recorder (Nihon Kohden, Medcorder), and the blood pressure was continuously monitored. Pulse rate was calculated from the blood pressure waves every 5th minute These parameters were measured also at the beginning of the infusions (=control values). Physiological saline or drugs diluted in saline were infused into V jugularis with constant rate of 0 13±0 003 ml/min (Harvard Syringe Pump model 2681). Some of the rats were killed at 10 min, the rest at 30 min. After the sample for hematocrit was taken, blood was centrifuged and serum stored at −18°C until

RESULTS AND DISCUSSION

The effect of various drugs and bleeding on the systolic (A) and diastolic blood pressure (B), the cardiac rate (C) and the serum TSH concentration (D) is shown in Fig. 1.

It became clear from these results that a rapid and profound decrease of the blood pressure as induced by the peripherally acting drugs like Na-nitroprusside and dihydralazine did not significantly depress the TSH secretion, although blood-flow in the pars distalis of the pituitary gland should be increased (Porter et al. 1967). The bleeding of the animals caused only a transient hypotension and—after a correction on the basis of the hematocrit—was not associated with any significant fall in the serum TSH levels. The monitoring of hematocrit is important if multiple blood samples are collected for hormone analysis.

The effects of drugs increasing the blood pressure on the serum TSH concentrations were not consistent and with one exception (noradrenaline i.p. at 30 min.) did not reach statistical significance.

The pentobarbital anesthesia may contribute to the rather small fluctuations of the serum TSH concentration. We have shown earlier that although various anaesthetics only slightly modified the basal TSH levels, they nearly completely inhibited the cold-induced TSH secretion (Männistö et al. 1976). Therefore the studies on anesthetized animals cannot directly be compared with those performed on intact animals.

It is concluded that in spite of the minor correlation (r 0.4502) between the change of the blood pressure and the change of the serum TSH concentration, the drugs modifying the TSH secretion do not exert their action solely by changing the blood pressure of the experimental animals.

We are grateful to the NIAMDD, the Rat Pituitary Hormone Distribution Program for providing the rat TSH RIA kit. This study was supported by the grant from the Academy of Finland and Signe och Ane Gyllenberg Stiftelse, Helsinki, Finland. The technical help of Dr Jyrki Mattila is greatly appreciated.

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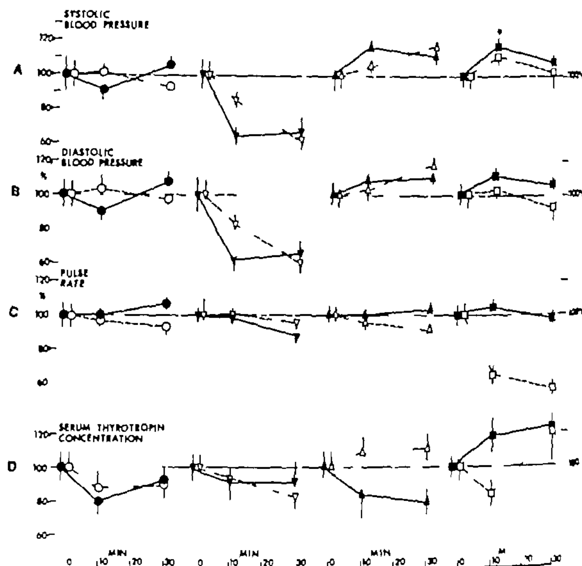


Fig 1 Effect of the intravenous infusion of 0 9% saline O- -O dihydralazine ▽ ▽ Na-nitroprusside ▼—▼ angiotensin amide △ △ and noradrenaline ▲—▲ or bleeding ●—● or the intraperitoneal injection of clonidine 1 mg/kg □ -□ or noradrenaline 1 mg/kg ■—■ on the systolic (A) diastolic (B) blood pressure pulse rate (C) and the serum TSH concentrations (D) The values are expressed as % of the preinfusion values which were in the following range systolic blood pressure 135–165 mmHg diastolic blood pressure 90–120 mmHg pulse rate 400–480/min and serum TSH level 360–530 ng/ml Mean ±S E n=6–7 The statistical significance of the change from the preinfusion values is shown as follows =$P<0.05$ =$P<0.01$ =$P<0.001$

analysed for serum TSH concentration by a homologous radioimmunoassay as described earlier (Ranta 1975) Some rats were injected i p. with clonidine or noradrenaline (1 mg/kg) and killed at 10 or 30 min Blood pressure cardiac rate and serum TSH concentration were monitored and saline infused as above Some rats were bled (about 5 ml) at 0 time and the parameters were followed as above Several intact rats (not cannulated unanesthetized) were also killed throughout the experiments.

The following drugs were used Angiotensin amide (Hypertensin® Ciba, Basle) 0 015 mg/ml the total dose 0 018 mg in 10 min 0 055 mg in 30 min Clonidine HCl (Orion Helsinki) 1 mg/kg i p Dihydralazine mesylate (Nepresol Ciba Basle) 0.3 mg/ml 0.37 mg in 10 min 1 11 mg in 30 min Noradrenalin bitartrate (Nor Adrenalin® Star Tampere) either 1 mg/kg i p or an infusion 0 015 mg/ml 0 018 mg in 10 min 0 055 mg in 30 min Sodium nitroprusside (Nipride Roche Basle), 0 1 mg/ml 0 1 mg in 10 min 0.37 mg in 30 min. The doses of drugs refer to respective acids or bases

Blood pressure and cardiac rate are expressed as a percentage of the preinfusion pressure and cardiac rate respectively Serum TSH concentration is expressed as a percentage of the TSH concentrations of the intact rats The serum TSH levels were corrected on the basis of the change in hematocrit The statistical significance of the changes induced by the various treatments was tested by using Student t test and in case of serum TSH concentration also analysis of variance was used The relation between the change of the blood pressure and the change of the serum TSH concentration was tested with Pearson s regression analysis

Acta Physiol Scand 1979 107 109–114

Morphometry of myocardial apex in endurance-trained mice of different ages

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KAINULAINEN H. PILSTRÖM L. & VIHKO V Morphometry of myocardial apex in endurance-trained mice of different ages. Acta Physiol Scand 1979 107 109–114 Received 22 Feb 1979 ISSN 0001-6772. Department of Cell Biology University of Jyväskylä, Finland and Institute of Zoophysiology University of Uppsala, Sweden

Mitochondrial volume density surface density of the outer mitochondrial membrane the mean number and size of mitochondria, and the mean surface density of crista membranes together with the volume densities of myofibrils and sarcoplasmic space were morphometrically analyzed in cardiac muscle of two groups of sedentary control mice aged 3 and 7 months, and in two groups of mice trained either 1 month rather intensely or 4 months moderately Of the calculated mitochondrial variables only the surface density of the outer mitochondrial membrane differed between the older controls and the older trained animals, the density being slightly smaller in the trained group The myofibrillar volume density of the older controls was smaller than that of the younger controls, while the sarcoplasmic volume density was larger The latter difference possibly function of age, was also observed in the trained groups. The result suggest that at   certain steady state level of exercise-induced cardiac muscle hypertrophy the muscle cells of trained mice do not differ markedly in ultrastructural properties from those of sedentary controls.

Key ords Stereology endurance training, cardiac muscle mitochondria, mouse

The effects of endurance training on skeletal and cardiac muscle are different Endurance training causes typical adaptation in the energy metabolism of skeletal muscle These adaptations are centred on the aerobic component of the energy-producing mechanism of muscle fibres and have the effect of making skeletal muscle more reminiscent of cardiac muscle (Holloszy & Booth 1976, Pette 1966) They include increased enzyme activities of β-oxidation (Mole et al 1971) citric acid cycle (Holloszy et al 1973), and the respiratory chain (Baldwin et al. 1977) Ultrastructural tudies show increased mitochondrial olume density (Hoppeler et al. 1973 Kiessling et al 1974 Vihko et al 1975) Endurance training does not usually cause either fibre hyper trophy or changes in total protein content (Holloszy & Booth 1976)

The most typical effect of endurance training on myocardium i an increase in weight (Arcos et al. 1967 Bozner & Meessen 1969 Crews & Aldinger 1967 Oscai et al. 1971a b). No major enzymatic adaptations have usually been found and the few ultrastructural studies regarding the effects of endurance training on cardiac muscle especially on its mitochondria, have yielded contradictory results (Bozner & Meessen 1969 Gollnick et al 1971 Pardaqua et al 1977) It therefore appears that myocardium responds to long-term physical exercise by hypertrophy (Bozner & Meessen 1969 Sohal et al. 1968) and that no major qualitative or quantitative adaptations comparable to those in skeletal muscle occur in energy-producing mechanism of trained cardiac muscle cells.

The aim of the present investigations was to study by stereological methods the effects of two types of endurance training on the ultrastructure of mouse cardiac muscle A relatively short lasting but intense and a longlasting, moderate running programme on a motor-driven treadmill were used Using this experimental design limited data were also obtained on the effects of training on the myocardium of mice of different ages

oom temperature The muscle pieces were then transferred for 2 h into 7.5% saccharose buffered with 0 1 M odium cacodylate, after which they were postfixed with % osmium tetroxide for h at +4°C. The pieces were hen immersed for 3 min in veronale buffer (pH 7 4 oom temperature) containing 7.5% saccharose and after hat were dehydrated for 3 5 min in 70 and 96% ethanol and for 3 5 min in 100% ethanol The dehydrated samples were transferred to propione oxide for 3 7 min and hen to propylene oxide Epon 812 (1 1) mixture for 1 h, after which they were embedded in Epon (A B 1 ... DMP 0.2) Samples were kept at room temperature overnight and the Epon was then allowed to polymerize for 48 h at +60°C

Ultra thin sections ere cut with Porter-Blum ultramicrotome using glass knives The sections were mounted on 200 mesh grids, stained with lead citrate and examined and photographed with a JEM 100 U electron microscope

Morphometry

5 blocks were analyzed from each apex. Thin sections were cut at two different levels, separated by at least 0 5 mm. These sections were mounted on two different grids. Thus two grids per apex were examined. The necessary number of micrographs was estimated as described by Weibel & Gomez (1968) and by Herbener et al (1973) to be approximately 20 (Fig. 2) Accordingly two micrographs per grid were taken resulting total of 20 prints per myocardium

Morphometrical volume densities were determined by the point-counting method (e.g. Weibel 1969) and outer mitochondrial and crista membrane areas using curved line grid superimposed on each micrograph (Merz 1968 Weibel 1969) The point-counting lattice included 80 points and the length of the curve as 301 cm at final magnification (Fig 1) The mean number of mitochondria is calculated according to the method of Weibel & Gomez-Duran (1962) Mean mitochondrial volume was obtained by dividing the mitochondrial volume density by

...m³)	V... (...m)	S... (m²/cm³)
45+0 04	48 15±2 12	9 03±2.00
34+0 06	50 40±1 85	8 91±0.91
34+0 04	48 59±1 66	8 77±1 43
31 0 03	47 88±1.67	8 10+1 49

the number of mitochondria. The surface density of the crista membranes of each apex was estimated from 4 micrographs including at least 200 mitochondria, with a total magnification of 22 830 The other morphological variables were estimated from micrographs with total magnification of 13 650 The abbreviations for variables used are given in Table 1

The data obtained from the stereological analyses were tested by the Student' *t*-test.

RESULTS

The morphometrical results are given in Table 1 Morphologically the ultrastructure of myocardial apex appeared normal in every group of mice Very few statistically significant differences were found between groups.

The mitochondrial volume density the surface density of crista membranes, and the mean number or mean size of mitochondria did not differ between the groups. Almost half of the muscle volume was occupied by mitochondrial mass in the myocardial apex Depending on the primary magnification used this value varied between 43 and 50% (Table 1)

The surface density of the outer mitochondrial membrane was slightly lower ($P<0.01$) in the trained TR2 group than in the corresponding control (C2) group The mean myofibrillar volume density of all animals was slightly lower (43.23%) than the volume density of mitochondria (44.08%) The myofibrillar volume density in group C2 was a little smaller ($P<0.05$) than that in group C1 The sarcoplasmic volume density was consequently larger ($P<0.05$) in group C2 than in group C1 In the trained groups a similar difference was also observed sarcoplasmic volume density in group TR2 was larger ($P<0.01$) than in group TR1

There were no significant differences in the olume densities of the extracellular space between the different groups (Table 1)

DISCUSSION

It is generally accepted that morphometrical methods although rather laborious offer a reliable means of carrying out quantitative studies on subcellular structure (e.g. Weibel 1969 Herbener et al 1973 Hoppeler et al 1973 Kiessling et al. 1974 Pantagna et al 1977) They are especially useful in comparative studies and in studies in which the effects of a certain treatment are traced

In term of its mitochondrial variables the apex



Fig. 1. The lattice used for the estimation of mitochondrial volume and surface densities. 80 points were used for the volume densities and point-counting was performed with total magnifications of 13 650× and 22 850×. The curved line was used for the crista membrane surface densities with a magnification of 22 850× and for the mitochondrial outer membrane density determinations with a magnification of 3 650×; the total length of the line being 301 cm. The size of the lattice was 19.1×23.8 cm. The sample in the figure was taken from a control animal of group C1.

MATERIAL AND METHODS

Animals and training

F-hybrid mice of the strain B6D₂F1/BOM (G. L. Bomholtgård Ltd, Ry, Denmark) aged 60 (53–67) days at the beginning of the experiment were used. The mice were randomly divided into two training groups (TR1 and TR2) exercising regularly and into two sedentary control groups (C1 and C2). Controls and the training mice, except while training, lived under ordinary cage conditions with free access to solid food pellets (Hankkija, Finland) and tap water. The training in the TR1-group was intense. These mice ($n=9$) were made to run on a motor-driven treadmill with horizontal tracks daily for 30 min at a speed of 20 m/min and for 45 min at 18 m/min before noon, and for 75 min at 18 m/min in the afternoon. This training programme lasted for 30 days and the exercises were performed 7 days a week.

In the training group TR2 ($n=10$) training was moderate but lasted for a longer period than that in the TR1-group. The programme consisted of a run on the treadmill for [illegible] min at a speed of 20 m/min 5 days a week over a period of 120 days.

Two trained animals and two controls were killed the day after the last exercise during each experiment day. Animals in the first control group (C1) ($n=9$) were thus of the same age as the animals in the first training group (TR1) and the animals in the second control group (C2) ($n=10$) were as old as those in the second training (TR2) group. The age difference between the younger and older animal groups was 3 months.

Electron microscopy

Mice were killed by dislocation of the neck and the heart was immediately removed. The apex of each myocardium was cut off and immersed in ice-cold 2% glutaraldehyde buffered with 0.1 M sodium cacodylate at pH 7.4. The tissue samples were cut into small (approx. 1 mm³) pieces and left in the fixative for 24 h at +4°C and then for 1 h at

Table 1. *Results of morphometrical analysis of myocardial apex in two groups of sedentary control mice and in two groups of endurance trained mice. Mean ± S.D.*

Data in the left part of the Table are calculated from micrographs taken with primary magnification 4000 × and in the right part with primary magnification 6700 ×. $V_{V,mit}$ = volume density of mitochondria, $V_{V,mf}$ = volume density of myofibrils, $V_{V,sp}$ = volume density of sarcoplasmic space, $V_{V,ec}$ = volume density of extracellular space, $N_{V,mit}$ = average number of mitochondria, S_V = surface density of outer mitochondrial membrane, [illegible] = average size of mitochondria, $S_{V,cr}$ = surface density of cristae

Group	Age (months)	Volume densities $V_{V,mit}$	$V_{V,mf}$	$V_{V,sp}$	$V_{V,ec}$	$N_{V,mit}$/ 10 μm	$S_{V,mit}$ (m²/cm³)
C1	3	43.74±1.43	44.36±1.15	9.16±0.64	2.7[illegible]±0.[illegible]0	5.24±0.29	2.06 0.[illegible]
C2	6	45.18±1.05	40.95±0.68*	11.53±0.86*	[illegible]33±0.[illegible]4	4.93±0.[illegible]0	2.10 0.04
TR1	3	43.81±1.00	44.95±0.87	9.17±0.52	2.09±0.[illegible]6	4.76±0.31	[illegible]01 0.[illegible]
TR2	6	43.59±1.01	4[illegible].66±0.76	11.6[illegible]±0.61	[illegible].20±0.[illegible]	4.65±0.09	[illegible]00 0.0[illegible]

Statistically significant ($P<0.05$) difference compared to group C1.
Statistically significant ($P<0.01$) difference compared to group TR1.
Statistically significant ($P<0.01$) difference compared to group C2.

tochondrial number and mass after long-term exercise

Endurance training did not cause any major qualitative changes in mitochondria. The surface density of cristae was similar in every animal group suggesting unchanged oxidative capacity in the trained myocardium. Pantagua et al (1977) reported no significant changes in the amount of mitochondrial protein nor in the specific activities of malate dehydrogenase, cytochrome c oxidase and ATPase in rat myocardium after swimming training. Myofibrillar and mitochondrial volume densities and the number of mitochondria were also unchanged in their study. In the present study a small decrease due to training was recorded in the surface density of outer mitochondrial membrane. The results of Edington & Cosmas (1972) and Cosmas & Edington (1975) showed a decrease in the average volume of mitochondria resulting in an increased outer surface area, if the volume density remains unchanged. In the present study the mitochondrial volume density and the average number of mitochondria per unit volume were slightly smaller in the TR2 group than in the C2 group, resulting the same average volume (0.94 μm³) of a mitochondrion in both groups. Evidently the difference in the surface density of outer mitochondrial membrane is caused by the slightly smaller average number of mitochondria in trained muscle.

The volume density of myofibril was larger in the younger C1 than in the older C2 control group. By contrast, the volume fraction of sarcoplasm (in this study all other cellular contents than mitochondria, myofibrils and nuclei) was larger in the C2 than the C1 group. The latter difference was also observed in the trained animals. It is possible that this difference is due to the age difference between the groups.

The ventricle, all muscles, especially those of the left ventricle, might be better objects for ultrastructural studies concerning exercise-induced cardiac muscle hypertrophy. However, the present results from myocardial apex lend confirmation to earlier observations of the proportionality of mitochondrial biogenesis to hypertrophy in situations in which the stimulus causing the hypertrophy has acted for a relatively long time (Bozner & Meessen 1969, Meerson 1975, Meerson & Breger 1977). The early phases of endurance training programme before the hypertrophied cellular steady state has been attained might yield more information concerning the mechanisms of exercise-induced myocardial hypertrophy.

We thank Raija Vassinen and Paavo Niemanen for excellent technical assistance.

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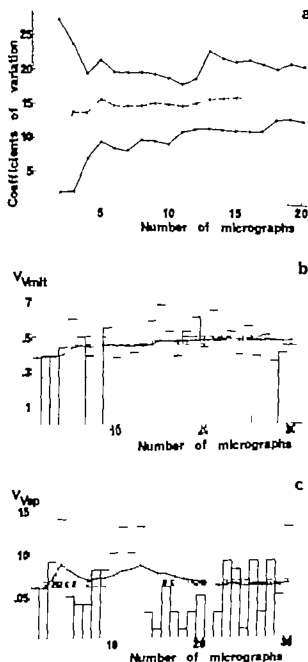


Fig (A) Determination of the requisite number of micrographs per muscle sample according to Herbener et al (1973) The broken line represent the verage value for the variation coefficient and the two unbroken lines the extreme values of the coefficient Data are taken from the mitochondrial volume densities in group C1 (B) Determination of the requisite number of micrographs for estimation of mitochondrial volume den ity Columns represent the volume density estimated per micrograph the fraction line represent the average value of the volume density as a function of increasing number of micrograph and the two lines of dashes indicate the 5% confidence limits of the mean volume density estimated from 30 micrographs. V_{Vmit} = volume density of mitochondria. (C) Determination of the requisite number of micrograph for estimation of the volume density of sarcoplasmic space V_{Vsp} = volume density of sarcoplasmic pace other e planations as in Fig 2B

of the myocardium seems to be between the atr and ventricular parts of mouse heart muscle The values of mitochondrial volume density measu in the apex in the present study were rather simil to those reported by Herbener et al (1973) in the ventricle muscle of mouse heart In the atrial pa of the myocardium the mitochondrial volum density reported by Herbener et al (1973) wa smaller (33–35%) than that in the apex (41–45 in this study Estimations of the mitochondrial volume density based on micrographs taken the more powerful magnification gave even high values (48–50% Table 1) as observed earlier (Ko et al 1976) This is partly due to the method of choosing such areas for electronmicrograph which contained large numbers of mitochondria t facilitate the analysis of the surface density of cristae

In respect of the number of mitochondria per unit volume of myocardium the apex (4 65–5 47 μm^3) was also between the atrium (5 69–7 77) and the ventricle (? 01–4 10) (Herbener et al 1973) The volume of an average mitochondrion wa greate in the ventricle (1 45–2 83 μm^3) medium in the apex (0 85–0 94) and smallest in the atrium (0 56–0 80)

No significant effects of training were observed in the indices of mitochondrial volume density number or average volume This is in agreement with the previous results of Arcos et al (1967 Bozner & Meessen (1961) Gollnick et al (1971) and Paniagua et al (1977) observed with rats A training programme consisting of 1 h daily running at a speed of 20 m/min for 5 days a week caused an 8–12% significant increase in the heart weight of mice after 20 exercises (Vihko & Salminen un published) The lack of training effects in mitochondrial variables or in myofibrillar volume density therefore suggests that myofibrillar or mitochondrial biogenesis occurs in proportion to cardiac muscle hypertrophy According to Bozner & Meessen (1969) mitochondrial volume fraction increases at early phases of training being at its highest on the fourth day after the beginning of training Therefore it i also possible that at early phases of hypertrophy mitochondrial capacity first increases and improved capacity for energy production enables rapid protein biosynthesis resulting in a new hypertrophied steady state relatively soon e g after one month of training as in this study (TR1-group) However Aldinger & Sohal (1970) and Arcos et al (1967) have reported increased

cta Physiol Scand 1979 107 115–126

Villous tissue osmolality and intestinal transport of water and electrolytes

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HALLBÄCK D A JODAL, M SJÖQVIST A. & LUNDGREN O Villous tissue osmolality and intestinal transport of water and electrolytes. Acta Physiol Scand 1979 107 115–126. Received 28 Febr 1979 ISSN 0001-6772. Department of Physiology University of Goteborg, Sweden.

The villous tissue hyperosmolality created by the intestinal countercurrent multiplier has been proposed to be of importance for fluid transport across the intestinal epithelium in vivo. This study was performed to test this hypothesis. Net transport of fluid and electrolytes (sodium, potassium and chloride), as well as unidirectional fluxes of water and sodium were determined in the small intestine of the cat. The villous osmolality was altered by varying the composition of sodium and glucose in the isotonic solutions perfusing the intestinal lumen. Net transport of fluid was correlated to tissue osmolality mainly due to an increase of the unidirectional flux of water from lumen to tissue with augmented tissue osmolality The results are thus consistent with the view that the intestinal countercurrent multiplier is of essential importance for net water transport. A correlation was found between net water and net sodium intestinal transport. A similar correlation was also demonstrated between net sodium and net chloride absorption rates in the jejunum while in the ileum net loss of sodium into the intestinal lumen was not accompanied by any corresponding loss of chloride loss This observation suggests the presence of sodium independent transport mechanism for chloride in the ileum but not in the jejunum.

The vascular arrangement in the cat intestinal villi is consistent with the existence of a countercurrent exchanger as also supported by experimental observations (for reviews, see Lundgren 1967 1974) Jodal and co-workers (Haljamae et al 1973 Jodal 1973 Jodal et al 1978) proposed that a villous countercurrent exchanger would be particularly important for water and solute absorption by creating a hyperosmolar compartment at the villous tip as a result of its countercurrent multiplying effects It was recently possible to quantify this hyperosmolality with a cryoscopic method (Jodal et al 1978) which revealed a villous tip osmolality beyond 1 000 mOsm/kg H_2O in the feline gut during luminal perfusion with an isotonic solution containing sodium and glucose

The efficiency of countercurrent multiplier is among other things dependent on the rate of active solute uptake By varying the composition of the luminal perfusate, it is possible to vary the rate of active absorption of solutes (e.g. sodium) which influences the concentration gradient between the two limbs of the countercurrent exchanger This, in turn affects the degree of tissue hyperosmolality at the villous tips (Jodal et al 1978 Hallbäck et al 1979*b*)

The present study was designed to investigate the effects on water and solute absorption when tissue osmolality in the intestinal villi was varied The efficiency of the countercurrent multiplier was altered by changing the sodium and/or glucose contents of the luminal perfusate Bidirectional fluxes of water and sodium as well as net potassium and net chloride transport were determined and correlated to tissue osmolality

METHODS

The experiments of this study were included in those recently reported by Hallbäck et al (1979*b*) Consequently operative procedures, recordings of net water transport, the cryoscopic technique and the solutions used are identical, and described in the mentioned paper The determi-

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Table . Absorption of water (net and fluxes) and the corresponding tissue osmolality in various portions of intestinal villi during the different experimental procedures of this study

Positive values for net absorption means absorption, negative values secretion. Mean values ± S.E.

	Net water movement (µl/min 100 cm²)	Water flux from lumen to tissue (µl/min 100 cm²)	Water flux from tissue to lumen (µl/ml 100 cm²)	Villous tip osmolality (mOsm/kg H_2O)	Mean osmolality (mOsm/kg H_2O) in upper 5 to 30% of villous length	5 to 90% of villous length	5 to 100% of villous length
Jejunum							
Krebs-glucose (n=9)	344+69	1 114±157	791±116	1 022±30	689±23	590±23	461±13
Krebs-mannitol (n=8)	221±59	1 065±182	830±109	901±50	652±34	580±28	468±15
Choline-glucose							
25 mmol Na (n=8)	77±33	822±177	742±108	701±77	560±47	497±38	421±25
Choline-mannitol							
25 mmol Na (n=9)	46±35	705±75	659±74	633±54	518±35	475±27	410±15
Choline-glucose (n=9)	−40±26	1 141±75	1 182±65	631±64	491±41	448±33	395±21
Choline-mannitol (n=6)	−97±32	779±206	867±101	495±58	430±43	405±37	368±[illegible]
Choline-maltose (n=6)	20±42	737±129	717±107	579±37	463±21	428±17	383±12
Ileum							
Krebs-glucose (n=8)	252±6[illegible]	931±178	713±163	940±33	679±22	591±19	478±15
Krebs-mannitol (n=8)	188±51	1 110±129	905±119	895±57	680±43	601±34	482±19
Choline-glucose							
25 mmol Na (n=9)	109±32	779±97	670±[illegible]	764±53	603±36	535±28	437±15
Choline-mannitol							
25 mmol Na (n=9)	3±32	597±72	565±59	643±12	545±13	501±11	430±8
Choline-glucose (n=8)	43±79	776±138	734±125	629±34	513±19	466±16	403±10
Choline-mannitol (n=6)	−86±53	434±1[illegible]1	539±122	463±67	399±43	381±34	343±19
Choline-maltose (n=6)	−6[illegible]+65	561±67	623±72	658±46	554±50	504±48	431±37

...as zero in both the jejunum and the ileum. The studies of unidirectional fluxes (Fig.) revealed that the increased rate of net water absorption along with augmented tissue osmolality was largely explained by a marked increase of lumen to tissue flux of water (about 410 and 490 $\mu l/min \times 100\ cm^2$ in jejunum and ileum respectively, right panel of Fig.) in both intestinal segments. The corresponding

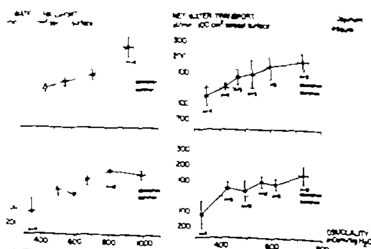


Fig. 1. The relationship between intestinal net water transport and villous tip osmolality (left panels) or mean tissue osmolality in the upper 5 to 30% of villous length (right panels) in jejunum (upper panels) and ileum (lower panels). The results obtained with the different perfusates were classed according to tissue osmolality. Bars denote S.E.

nations of water and electrolyte fluxes will be described below

A Biochemical and radioactive measurements

To determine water and electrolyte fluxes across the intestinal epithelium, samples (?–?.5 ml collected in plastic tubes) were taken of the perfusate entering and leaving the intestinal segments after a perfusion period lasting at least ?5 min Sodium and potassium concentrations of these samples were measured by flame photometry (Eppendorf) and chloride concentrations with a chloride meter (Corning Eel 920 Chloride meter Halstead Essex England)

To determine unidirectional water and sodium transport from lumen to tissue 50–60 μCi HTO and 4–8 μCi ^{22}Na (0 05 μg) were added per liter perfusate and the β-radiation was estimated in a Packard Tri-carb liquid scintillation spectrometer The time of counting was prolonged so that the coefficient of variation was below 1% An external γ-standard was used to correct for quenching All analyses were carried out in duplicates

In 20 animals the arterial concentrations of the two tracers were determined at the end of the expt They never exceeded 3% of the luminal concentration In most expts they were about 1% of that of the perfusate

B Calculation

1 *Intestinal net water transport* was calculated from the continuously recorded change in perfusate volume in the recirculating system (see Jodal et al 1975)

2 *Intestinal net transport of electrolytes* were calculated according to the following formula

$$J_{net}=C_{in}\times V_{in}-C_{out}(V_{in}-J_{net}^{H_2O}) \quad (1)$$

For explanation of symbols used see Table 1

3 *Unidirectional water and sodium transport* were calculated according to Levitan et al (196?) and Skadhauge (1969) modified to fit our direct net water transport determinations Mean luminal concentrations of sodium and tracers (^{22}Na and HTO) were calculated assuming logarithmic reductions along the intestinal segment The following formulas were used

$$J_{ms}^{H_2O}=\frac{[HTO_{in}]\times V_{in}-[HTO_{out}](V_{in}-J_{net}^{H_2O})}{10^{\frac{1}{2}(\log[HTO_{in}]+\log[HTO_{out}])}} \quad (2)$$

$$J_{ms}^{Na}=\frac{[^{22}Na_{in}]\times V_{in}-[^{22}Na_{out}](V_{in}-J_{net}^{H_2O})}{10^{\frac{1}{2}(\log[^{22}Na_{in}]+\log[^{22}Na_{out}])}}\times 10^{\frac{1}{2}(\log[Na_{in}]+\log[Na_{out}])} \quad (3)$$

$$J_{sm}=J_{ms}-J_{net} \quad (4)$$

The fluxes were related to serosal area and expressed as μl/min × 100 cm² serosal surface (water) or μmol/min × 100 cm² serosal surface (electrolytes)

C Statistics

Statistical significance was tested using Wilcoxon's nonparametric tests A *P* value of 0 05 or less was considered as significant (Siegel 1956)

RESULTS

Water transport Table 3 summarizes the results of net and unidirectional water fluxes when the intestinal mucosa was exposed to the 7 different perfusates (see Table 1 Hallbäck et al 1979*b*) Table also gives the tissue osmolality data recorded with the different perfusates These results are presented as villous tip osmolality and mean tissue osmolality in upper 5 to 30 5 to 50 and 5 to 100% of villus length

Fig 1 gives rates of net water transport in jejunum (upper panels) and ileum (lower panels) plotted against villous tip osmolality (left panels) and against mean tissue osmolality in the upper 5 to 30% of villous length (right panels). The results are classed according to tissue osmolality regardless of perfusate used In fig 2 the unidirectional water transport rates are plotted in a similar manner

In Fig 1 negative values denote net water transport from tissue to lumen and positive values indicate net transport in the opposite direction Both in jejunum and in ileum net water transport changed from secretion to considerable absorption when tip or mean osmolality in upper 5 to 30% of villous length was enhanced and transport tended to level off with increasing osmolality At villous tip osmolalities around 500 mOsm/kgH$_2$O or at a mean tissue osmolality around 400 mOsm/kgH$_2$O in upper 5 to 30% of villous length net water transport rate

Table 1 *Explanations of symbols used in formulas 1–4*

J_{net}	net transport
J_{ms}	unidirectional transport of fluid or solute from lumen to tissue
J_{sm}	unidirectional transport of fluid or solute from tissue to lumen
V_{in}	rate of perfusate entering the intestinal segment
C_{in}	concentration of solute in the solution entering the intestinal segment
C_{out}	concentration of solute in the solution leaving the intestinal segment
$[Na_{in}]$, $[Na_{out}]$	concentration of sodium in the solution entering or leaving the intestinal segment
$[^{22}Na_{in}]$, $[^{22}Na_{out}]$	concentration of ^{22}Na entering or leaving the intestinal segment
$[HTO_{in}]$, $[HTO_{out}]$	concentration of tritiated water entering or leaving the intestinal segment

Table 3 Net sodium absorption as well as sodium flux from lumen to tissu and from tissue to lumen c aned ith the different perfusates used in the study

Positive values denote absorption negative secretion. Mean values ± S.E

	Net sodium movement (μmol/min 100 cm²)	Sodium flux from lumen to tissue (μmol/min 100 cm²)	Sodium flux from tissue to lumen (μmol/min 100 cm²)
num			
cbs-glucose (6)	49 2±13.6	91.0±13.0	46.6±5.5
rebs-mannitol (7)	6..6±16 3	79 7±15.9	77 7±7 1
tolme-glucose 25 mmol Na (=6)	10 8± 4 1	17.8± 5 7	30 1±2.7
tolme-mannitol 25 mmol Na (=7)	−1..9± 6.9	11.2± 1 4	4 1±7.3
tolme-glucose (=9)	−26 0± 9 2		
tolme-mannitol (7)	24.8± 5.2		
tolme-maltose (=6)	−28.0± 8 5		
um			
rebs-glucose (n 8)	38.5±11 1	100.5±15.3	6.. 0±16 4
rebs-mannitol (=5)	7' 6±12.9	105 0± 0 6	45.5±14 4
tolme-glucose 25 mmol Na (=8)	−5 1± 3.6	21 7± 5.5	27.2± 6.4
tolme-mannitol 25 mmol Na (=8)	17 7± 4 3	12.5± 1 7	30.2± 3 7
tolme-glucose (8)	−9.0± 2.8		
tolme-mannitol (6)	−25 8_ 9.9		
tolme-maltose (6)	26.9± 9 0		

nd no statistically significant difference could be emonstrated between the two groups.

In Fig. 5 the net rate of water transport is plotted s. that for sodium showing that this relationship almost linear in both jejunum and ileum. Addi- ion of glucose to the perfusates tended to increase he rate of water absorption in the jejunum though he effect was not statistically significant In the leum glucose addition had no effect.

Chloride transport Table 4 summarizes the measured rates of net chloride transport in the ejunum and the ileum. In Fig 6 net rat of chloride ransport is plotted vs. tissue osmolality in similar ay as for net water transport (Fig. 1) In the jeju- num the net rate of chloride transport was enhanced from secretion of 10 to an absorption of 30 μmol/ min 100 cm² serosal surface with increasing tissue osmolality A net secretion of chloride was re corded in 13 of the 19 expt with the lowest villous tissue osmolality In the ileum, on the other hand, secretion was only observed in 4 of the 19 xpts. ith the lowest osmolality In Fig 7 net rate of chloride transport is plotted vs. net rate of sodium transport. In the jejunum these two variables showed good correlation. In the ileum on the other hand, net sodium secretion was not accom- panied by any chloride los into the lumen.

Potassium transport The results on net potas- sium transport rate are presented in Table 4 and Fig. 8 In both jejunum and ileum a small absorption of potassium was seen in all the experimental series

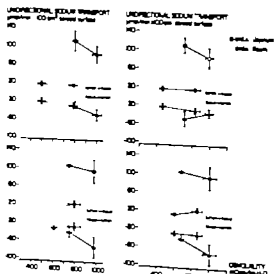


Fig 4 The relationship between unidirectional sodium transports and tissue osmolality Illustrated in the same way as Fig. 2 Results obtained ith solutions containing 25 mmol Na/l are depicted with dashed lines and those with 147 mmol Na/l solutions with solid lines Bars denote S E.

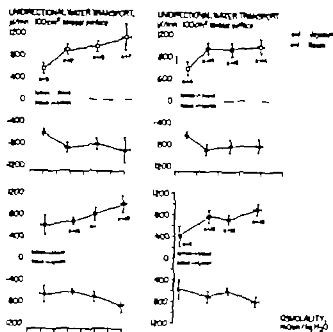


Fig 2 The relationship between the unidirectional transports of water and tissue osmolality The abscissa in the left panels denotes villous tip *osmolality and in the right* panels the mean tissue osmolality in the upper 5 to 30 % of the villi The upper panels show results from jejunum the lower panels from ileum The observations were classed according to tissue osmolality Bars denote S E

flux from tissue to lumen also showed a tendency to increase with enhanced tissue osmolality (about 190 and 250 μl/min×100 cm² in jejunum and ileum *right panel of Fig 2)* an effect that was most pronounced in jejunum at low osmolality

Sodium transport Data on sodium transport in jejunum and ileum are presented in Table 3 showing both rates of net sodium transport as well as rates of unidirectional sodium transport for the seven perfusates used negative signs indicating net sodium loss into the lumen

Fig 3 illustrates the compiled data on net sodium transport in jejunum (upper panels) and ileum (lower panels) plotted against villous osmolality The left panels show osmolality at the tip and the right panels mean tissue osmolality in upper 5 to 30 % of villous length Only experiments where the luminal perfusates contained 25 or 147 mmol Na are included and plotted separately

When the intestinal lumen was exposed to a perfusate containing 25 mmol Na/l net secretion occurred amounting to about 10 μmol sodium/min 100 cm² serosal surface This secretion tended to be constant despite varying tissue osmolality When the perfusate contained 147 mol Na/l there was net sodium absorption of 50–70 μmol Na/min×100 cm² serosal surface which appeared to be constant or even to decrease with increasing villous osmolality This decrease of net sodium transport was *not statistically significant*

Fig 4 summarizes the calculations on unidirectional sodium transport classified in the same manner as for net sodium transport It is evident that the difference in net sodium absorption between the 25 mmol and the 147 mmol Na solution (Fig 3) was mainly caused by a marked increase in sodium transport from lumen to tissue Transport in the opposite direction was fairly constant

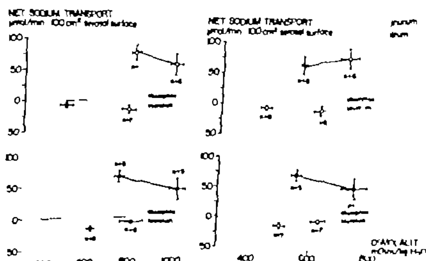


Fig 3 The relationship between net sodium transport and tissue osmolality Illustrated in the same way as in Fig 1 The results from the solutions containing 25 (dotted lines) or 147 mmol Na/l (solid lines) are included Bars denote S E

Table 3 *Net sodium absorption as ell as sodium flux from lume to tissue and from tissu to lumen ... th the different perfusates used in the study*

... tive values denote absorption, negative secretion. Mean values ± S.E.

	Net sodium movement (µmol/min 100 cm²)	Sodium flux from lumen to tissue (µmol/min × 100 cm²)	Sodium flux from tissue to lumen (µmol/min 100 cm²)
[illegible]			
...-glucose (6)	49.. ± 13 6	91.0 ± 13 0	46 6 ± 5.5
...-mannitol (7)	62 6 ± 16 3	79 7 ± 15 9	27 7 ± 7 1
...-glucose + 25 mmol N (6)	−10 8 ± 4 1	17.8 ± 5 7	30.1 ± 2.7
...-mannitol 25 mmol N (=7)	12.9 ± 6 9	11.2 ± 1 4	4 1 ± 7.3
...-glucose (=9)	−26 0 ± 9 2		
...-mannitol (7)	−24 8 ± 5.2		
...-maltose (=6)	28 0 ± 8.5		
[illegible]			
...-glucose (8)	38.5 ± 11 1	100 5 ± 15 3	6..0 ± 16 4
...-mannitol (5)	72.6 ± 12.9	105.0 ± 0 6	45.5 ± 14 4
...-glucose .5 mmol Na (8)	−5 1 ± 3.6	21 7 ± 5.5	77.2 ± 6 4
...-mannitol 25 mmol Na (=8)	−17 7 ± 4 3	12 5 ± 1 7	30.2 ± 3 7
...-glucose (n 8)	9 0 ± 2.8		
...-mannitol (=6)	25.8 ± 9 9		
...-maltose (6)	26.9 ± 9.0		

...d no statistically significant difference could be demonstrated between the two groups.

In Fig. 5 the net rate of water transport is plotted ... that for sodium, showing that this relationship ... almost linear in both jejunum and ileum. Addition of glucose to the perfusates tended to increase the rate of water absorption in the jejunum though the effect ... as not statistically significant. I... the ... glucose addition had no effect.

Chloride transport. Table 4 summarizes the measured rates of net chloride transport in the jejunum and the ileum. In Fig. 6 net rate of chloride transport is plotted vs. tissue osmolality in ... similar ...ay as for net water transport (Fig. 1). In the jejunum the net rate of chloride transport was enhanced from a secretion of 10 to an absorption of 30 µmol/min 100 cm² serosal surface with increasing tissue osmolality. A net secretion of chloride was recorded in 13 of the 19 expts. with the lowest villous tissue osmolality. In the ileum, on the other hand, secretion was only observed in 4 of the 19 expts. ...th the lowest osmolality. I... Fig. 7 net rate of chloride transport is plotted vs. net rate of sodium transport. In the jejunum these two variables showed ... good correlation. I... the ileum, on the other hand, net sodium secretion was not accompanied by any chloride loss into the lumen.

Pot... ...um transport. The result... on net potassium transport rate are presented in Table 4 and Fig. 8. In both jejunum and ileum a small absorption of potassium was seen in all the experimental series.

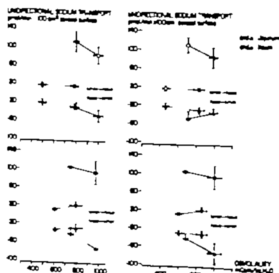


Fig. 4. The relationship between unidirectional sodium transports and tissue osmolality illustrated in the same way ... Fig. ... Results obtained with solutions containing 25 mmol Na/l are depicted ...ith dashed lines and those ...ith 147 mmol Na/l solutions with solid lines. Bars denote S.E.

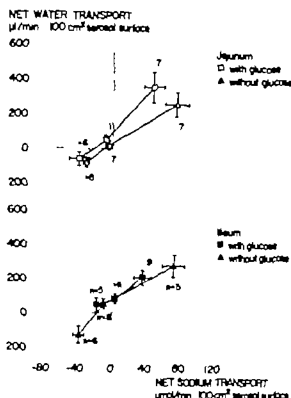


Fig 5 The relationship between net water transport and net sodium transport in jejunum (upper panel) and ileum (lower panel) Squares illustrate results obtained with solutions containing glucose and triangles depict experiments with glucose-free solutions. Bars denote S E

DISCUSSION

There is no evidence to suggest that water transport across epithelia is an active process It is generally assumed that water passively follows actively transported solutes (House 1974). To expla[in] water absorption in vivo in the absence of a lumen to plasma osmolar gradient, a hyperosmolar compartment has been postulated to exist in the intestinal mucosa (Curran & McIntosh 19.., Schultz & Curran 1968) which according to Schultz & Curran (1968) should be localized to the space between the epithelial cells and the basement membrane Diamond & Bossert (1967) on the other hand proposed that a standing hyperosmolar gradient is localized to intercellular spaces of epithel[ia]. Such hypertonic spaces around the epithelial cells have however never been experimentally demonstrated in vivo in the small intestine In vitro Machen & Diamond (1969) and Simmons & Na[f]tlin (1976) provided indirect support for the existence of a hyperosmolar compartment in the mammalian gall bladder and small intestine respectively Gupta Hall & Naftalin (1978) localized an extracellular hyperosmolar region between the epithelial cells by electron microprobing but the observed profiles of [Na] [K] and [Cl] around the enterocytes were not consistent with the standing gradient hypothesis of Diamond & Bossert (1967) or its modifications by Sackin & Boulpaep (1975).

An alternative hypothesis explaining water transport in vivo across the epithelia in villous structures was proposed in reports from this laboratory According to this hypothesis a countercurrent exchanger exists in most villous structure[s]

Table 4 Net absorption of chloride and potassium in jejunum and ileum during the different experimental procedures of this study

Positive values denotes absorption negative value secretion Mean values ± S E

	Net Cl movement (µmol/min × 100 cm²)	Net K movement (µmol/min × 100 cm²)
Jejunum		
Krebs-glucose	38.6±10 4 (=9)	0 69±0 48 (=8)
Krebs-mannitol	37 4± 8.6 (n=8)	46±0 78 (n=6)
Choline-glucose+25 mmol Na	18 8± 8 2 (n=9)	−0 69±1 23 (=9)
Choline-mannitol+25 mmol Na	−0.5± 6 5 (n=9)	0 77±0 25 (n=9)
Choline-glucose	−18 1± 8.5 (n=9)	0 1±1.20 (=10)
Choline-mannitol	−6.5± 9 (n=4)	0 83±0.59 (=6)
Choline-maltose	−[illegible]8± 7 9 (n=6)	−0 55±0 98 (n=6)
Ileum		
Krebs-glucose	57 7± 6.6 (n=9)	1 31±0.33 (=9)
Krebs-mannitol	47.3±14.6 (n=8)	4±0 45 (n=6)
Choline-glucose+25 mmol Na	13 4± 6 8 (n=8)	1 57±0.34 (n=9)
Choline-mannitol+25 mmol Na	1[illegible]± 9[illegible] (=9)	0 87±0 38 (n=9)
Choline-glucose	[illegible]3.5± 4.8 (n=8)	.56±0.33 (n=8)
Choline-mannitol	[illegible]3.5±11.8 (n=4)	1 6 ±0.56 (n=7)
Choline-maltose	0± 7 8 (n=6)	−0.20±0 30 (n=6)

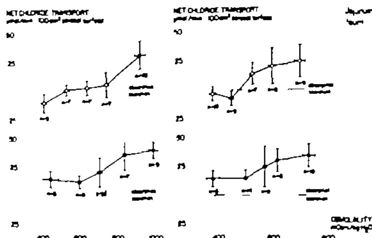


Fig. 6. The relationship between net chloride transport and tissue osmolality illustrated in the same way as in Fig. 1. Bars denote S.E.

functioning as a countercurrent multiplier to create marked increases in villous osmolality (cf. Haljamae et al. 1973, Jodal 1973, Jodal et al. 1978, Hallbäck et al. 1979*b*). Similar mechanisms have been proposed to exist also in papillary structures of the tongue (Hallbäck et al. 1979). The evidence for these mechanisms includes the demonstration of very high electrolyte concentrations (Haljamae et al. 1973, Zeuthen & Monge 1975) and marked hyperosmolality (Jodal et al. 1978, Hallbäck et al. 1979) in the villous core in vivo. A close correlation between rate of net water absorption and villous core osmolality is, however, a prerequisite for accepting this hypothesis and the present report is devoted to such an analysis.

The efficiency of a villous countercurrent multiplier and, hence, the extent of villus hyperosmolality depend on several variables, such as blood supply characteristics (including capillary permeability and blood flow), epithelial hydraulic conductivity, epithelial solute permeability, rate of active solute transport in the villi. In the present study tissue hyperosmolality was altered primarily by changing the lastmentioned variable, accomplished by varying the composition of the isotonic perfusates used to supply the intestinal lumen. Based on observations in both in vitro and in vivo experiments it is generally accepted that sodium is the main ion determining intestinal water absorption (Schultz & Curran 1968). It is also known that passage of sodium across the luminal border of the enterocyte is facilitated when glucose is present in the intestinal lumen (Crane 196., Schultz & Curran 1970). For such reasons the concentrations of sodium and glucose in the luminal perfusate were altered in the present expts.

The results presented in Fig. 1 show a close cor

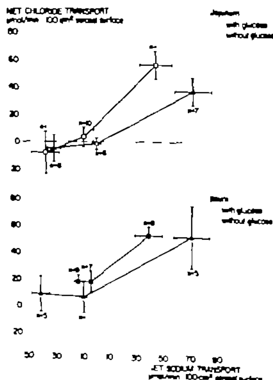


Fig. 7. The relationship between net chloride transport and net sodium transport in jejunum (upper panel) and ileum (lower panel). Squares depict results obtained with solutions containing glucose and triangles illustrate experiments with glucose-free solutions. Bars denote S.E.

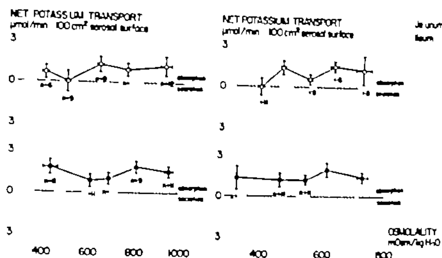


Fig 8 The relationship between net potassium transport and tissue osmolality illustrated in the same way as in Fig 1 Bars denote S E

relation between net water transport and tissue osmolality Net water secretion changed into net absorption as villous osmolality increased from just above isotonicity to a marked hyperosmolality This increase in villous osmolality augmented primarily the flux rate of water from lumen to tissue thus supporting the hypothesis that the villous countercurrent exchanger is crucial for intestinal water absorption

Assuming that the tissue osmolality measured in this study represents the only driving force for net water absorption it is possible to calculate a hydraulic conductivity coefficient of the intestinal epithelium (L_p) This assumption represents of course an oversimplification of the physiological situation It should however be pointed out that the osmotic forces created by the tissue osmolality are very large indeed as discussed in previous reports (Jodal et al 1978 Hallbäck et al 1978) The calculations presented in Table 5 are based on the net water absorption rates and the tissue osmolality measurements reported in Table _ The L_p values were calculated from the difference between the choline-mannitol group and the other groups and they are expressed per cm² mucosal surface using the ratios for mucosal to serosal surface area reported by Wood (1944) The reflection coefficient was set to 0 60 for the jejunum and to 0 85 for the

Table 5 *The diffusional permeability to water (P_d) and the hydraulic conductivity (L_p) in the jejunum and the ileum of the cat*

All calculations were made assuming that the exchange of water occurred across varying portions of the villi The details of the calculations are described in text

	Length (%)	Jejunum P (cm×s ×10⁴)	Jejunum L_p (cm× × Pa ×10¹¹)	Ileum P (cm×s ×10⁵)	Ileum L_p (cm×s × Pa ×10¹¹)
Krebs-glucose	30	2.6	36	1.8	23
	50	1 7	34	1..	20
	100	0.87	34	0 60	16
Krebs-mannitol	30	_6	31	1 8	18
	50	1 7	26	1..	15
	100	0 87	_2	0 60	1
Choline-glucose	30	_6	19	1 8	22
	50	1 7	17	1..	19
	100	0 87	14	0 60	14

um, since it was assumed that the tissue osmolali- ncrease was mainly made up of sodium chloride ordtran et al. 1965). Table 5 includes calculations L_p assuming that the water absorption takes ace along the whole villus or only at the upper 30 50% of the villi. When doing the calculations in e two lastmentioned groups the assumed muco- l surface areas were altered correspondingly. The values of this study are of the same order of agnitude as those reported in earlier investiga- ons on the small intestine in vivo (cf. House 1974). ompared with the L_p values for villous capillaries ported earlier ((20–45)×10⁻¹⁰ cm s⁻¹ Pa⁻¹ Biber et 1973), the hydraulic conductivity of the intestinal pithelium is much smaller.

It is also possible to roughly estimate the diffu- e permeability of water (P_d) from the present sults. For these calculations we have chosen the ean influx rate of tritiated water in the experi- ents with the lowest villous osmolality, i.e. when hoine mannitol was used as luminal perfusate Table). In these experiments diffusion ought to e the dominating process by which tritiated water oves into the tissue. The values obtained are gi- en in Table 5. The diffusive water permeability ecorded in the present study is similar to that noted other studies in vivo (House 1974).

The result presented in Fig. 5 on the relationship between net transport of fluid and net transport of sodium suggests a clear relationship between these two transport processes as has also been shown in a large number of other studies (In vitro: Curran 1960, Clarkson & Rothstein 1960, Barry et al. 1965 ... Curran & Salomon 1957, Phillips & Sum- merskill 1967, Fordtran et al. 1968, Sladen & Daw- son 1969).

The result of the present study indicate that addition of glucose to a perfusate containing 147 mmol NaCl caused no significant change in sodium transport in the jejunum or in the ileum. However addition of glucose to solutions containing 25 mmol NaCl enhance the mucosal to serosal flux of sodium. T different mechanisms have been proposed to explain the effect of glucose on the lumen to tissue transport of sodium. The sodium gradient hypothesis implies that sodium and glucose in- teract with common carrier-transport system at the apical border of the enterocyte and that both solutes are transported transcellularly (Crane 1962, Schultz & Curran 1970, Schultz & Frizzel 1972). On the other hand, in in vivo expt on humans Fordtran and coworkers (Fordtran et al. 1968, Turnberg et al. 1970, Fordtran 1975) obtained evi- dence in jejunum but not in ileum for a passive absorption of sodium via a solvent drag phenome- non secondary to an active glucose transport. It is not possible to differentiate between these two mechanisms on the basis of the present results. However the results do not support the proposal by Fordtran and collaborators that sodium trans- port is governed by different mechanisms in jejunum and ileum.

Although the present results do not suggest any difference for glucose transport between the jejunum and the ileum the observations on chloride transport clearly indicate that it differs in the two parts of the small intestine. Thus the results pre- sented in Figs. 6 and 7 and Table 4 suggest the presence of a sodium independent transport mechanism of chloride ions in the ileum while no such mechanism seems to exist in the jejunum. This observation corroborates earlier reports that have proposed the presence of a chloride–bicarbonate exchange mechanism in the ileum (Turnberg et al. 1970*b*) as well as a sodium dependent transport (Nellans et al. 1974). In jejunum no sodium inde- pendent mechanism can be detected either with the present experimental technique or by Turnberg et al. (1970*a*). The conclusions regarding a chloride– bicarbonate interaction in vivo are based on the observation of a high intraluminal P_{CO_2}. It should however be underlined that the high carbon dioxide concentration in the small intestinal lumen may at least in part, be produced by the presence of the intestinal countercurrent exchanger which very ef- fectively tends to delay the net absorption of lipophilic solutes such as CO_2 (*Lundgren 1967*, Hamilton et al. 1967, 1968, Bond et al. 1977).

Potassium is generally believed to be passively absorbed through solvent drag (for ref. see Turnberg 1972). Our study indicates that net potas- sium transport is rather constant in spite of a rising villous tissue osmolality and an increasing net water absorption. However an augmented passive potas- sium absorption by solvent drag into the intestinal tissue may be balanced by an increased loss of potassium from the epithelium cells into the lumen. This, in turn may be secondary to a rise in intracel- lular K⁺ produced by the increased, active pumping of potassium at the baso-lateral membrane by the Na–K dependent ATP-ase pump.

The hypothesis that a countercurrent multiplica-

tion of sodium provides the driving force for passive water and electrolyte absorption in the small intestine implies that the in vivo system is extremely complicated In a previous study the importance of different vascular parameters for the efficiency of the countercurrent exchanger was investigated (Jodal et al 1978) Furthermore experiments were performed by Hallbäck et al (1979*b*) which studied the dependence of villous tissue osmolality on varying the active solute transport The present study relates tissue osmolality to transport of water across the epithelium However to get a detailed knowledge of the interaction between the countercurrent multiplier and the transport of water and solutes across the intestinal epithelium the influence of a large number of other factors has to be evaluated

One important factor in this context is the ability of the enterocyte to transport solutes against considerable lumen to tissue concentration gradients The rate of active transport depends on a large number of factors such as the thickness of the unstirred layer in the lumen the intracellular concentration of the various solutes the number of pumping sites per unit plasma membrane surface area. The latter factor may in some instances vary along the villus due to an adaptation to the extracellular environment (cf Csaky & Fischer 1977) Furthermore the active transport of electrolytes together with the extracellular and intracellular ion concentrations determine the potential difference across the plasma membrane of the enterocytes one further driving force for epithelial movement of ionized solutes

The established tissue hyperosmolality forces water and dissolved solutes into the tissue through pores by most workers believed to be mainly located in the epithelial tight junctions The rate of fluid transfer in the paracellular routes is among other things determined by the width of this space and the leakiness of the tight junctions. The size of the intercellular space is in turn dependent on the interstitial hydrostatic pressure created by the presence of the tissue hyperosmolality according to Curran s double membrane hypothesis The rate of fluid transfer is also one factor that governs the thickness of the unstirred layer by varying the convectional stirring at the lumen-tissue interphase

It is important to realize that all the factors discussed above probably vary quantitatively along the villous length The situation is further com[illegible] cated by a descreasing oxygen tension and in[illegible] creasing carbon dioxide tension in the tissue [illegible] base to tip also a result of the intestinal counter[illegible] rent exchanger (Lundgren 1967) Finally the pr[illegible] able existence of active secretory mechan[illegible] perhaps mainly localized in the crypts have to [illegible] taken into the account as well as a diffusion [illegible] solutes into the lumen in the villi along favoura[illegible] concentration gradients

The description above clearly indicates that [illegible] large number of technically very difficult exper[illegible] ments are needed before a detailed understanding [illegible] the villous transport mechanisms in vivo are [illegible] tained and further that one should be cautious [illegible] transferring in vitro results to the in vivo situ[illegible] (cf Corbett et al 1977)

This research was supported by grants from [illegible] Swedish Medical Research Council (14X-2855), from [illegible] Swedish Society for Medical Sciences, from Harald [illegible] Greta Jeansson Fund from Magnus Bergvalls Stift[illegible] and from the Faculty of Medicine University [illegible] Göteborg

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fluence of intravenous infusion on heart rate sympathetic d vagal efferentation and left atrial and ortic baroreceptor activity in dogs

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The influence of 42 infusions of saline on heart rate sympathetic and vagal cardiac efferent activity and on the aortic baroreceptor and left atrial B-type nerve impulse activity was studied in 37 morphine-chloralose anesthetized dogs. The responses in heart rate were tachycardic in 31 infusions and bradycardic in 11 infusions. In tachycardia, sympathetic activity increased in majority of the cases but also decreases and nonsignificant changes were observed. Vagal efferentation decreased in most of the cases but also nonsignificant changes or increases in activity occurred. Sympathetic efferentation mainly decreased in bradycardic responses while vagal efferentation diverged in different directions in its nerve activity rate. The ratio of sympathetic to vagal impulses significantly correlated to the heart rate in most of the cases in tachycardia but not in bradycardia. It is concluded that sympathetic and vagal cardiac efferentation plays significant role in heart rate regulation in volume load-induced tachycardia but in bradycardia only the changes in sympathetic cardiac efferentation are important in respect to heart rate changes. The aortic baroreceptor and left atrial B-type receptor activity rate increased both in tachycardia and bradycardia. Changes in the activities of these receptors do not explain the different heart rate responses. It is supposed that bradycardic responses result from changes in cardiac contraction associated with some reflex mechanism suppressing the excitatory influence of the activity of atrial receptors on sympathetic cardiac efferentation.

Key ords autonomic nervous system, sympathetic efferent cardiac discharges, vagal efferent cardiac discharges, left atrial receptor discharges, baroreceptor discharges, heart rate

nce Bainbridge s (1915) observation on the influence of infusion on the heart rate in dogs numerous investigations have been done in order to investigate the phenomenon and to find the local ceptor area of the afferent pathway and the role of sympathetic and vagal efferentation in this reflex n these studies it ha been shown that a tachycardic response to infusion can be elicited Sawa & Miyazaki 1970, Anrep & Segall 1926) but not always (DeGraff & Sand 1925 Ball & Katz 1941), and it seem to depend on the control heart rate (Coleridge & Linden 1955 Pathak 1959 Jones 196_ Ahmed & Nicoll 1963 Horsch et al. 1964) and the grade of the local tretch (Goetz 1965). Also tachycardia can be elicited in denervated hearts (Pathak 1959 Donald & Shepherd 1963) The possible reflex area of afferentation has been studied by Ledsome & Linden 1964 Karim et al 1972 and Kappagoda, Linden & Snow 1972 and was found to be localized at the junction of the atria and blood vessels. Also in conscious dogs i v infusion induced tachycardia (Bishop & Peterson 1976) and the same reflexogenic pathways have been observed as in anesthetized dogs

The findings and observations on the activity in afferent and efferent cardiac nerves during i.v infusions or local distensions are sparse It has been demonstrated that atrial B-type activity and sym-

pathetic efferent cardiac activity increase during the distension of the atrial—blood vessel junction (Linden 1973) An increase in sympathetic cardiac efferentation during i v infusion has been reported by Bergström Hakumäki & Sarajas (1971) as well as a decrease in vagal efferentation and an increase in sympathetic cardiac activity (Hakumäki 1972) The difference between the left and right atrium has been studied by Koizumi et al (1975) who observed that distension of the right atrium always induced an increase in sympathetic efferent cardiac activity but on the left side the response to stretch was immediately a decrease and after that an overshooting in efferentation and heart rate In bled cats sympathetic afferent activity from the heart also increased after an injection of saline (Malliani et al 1975) Previous works have demonstrated that different types of local stimuli and also i v infusions induce both tachycardia and bradycardia and different types of responses in sympathetic and vagal efferentation while changes in afferentation and afferentation–efferentation relationships are unknown

The aim of this work was to study the function of left atrial receptors aortic baroreceptors and sympathetic and vagal efferent cardiac activity before during and after i v infusion in Bainbridge type experiments in anesthetized dogs in order to find out what kind of changes in afferentation and efferentation can be observed in cardio-acceleratory or bradycardic responses

METHODS

3? dogs weighing 8–15 kg were premedicated with morphine hydrochloride (1 mg/kg) and 1 h later anesthetized with an i v infusion of α-chloralose (100 mg/kg) The dog was then intubated and artificial respiration was started with a gas mixture of air and 20% oxygen When the chest was opened an expiratory resistance of 3 cm of water was placed in the expiratory outlet of the respirator The left side of the chest was opened and the fo rth rib was removed The upper lobe of the lung was resected and a paraffin oil pool wa formed inside the thorax ca ity from pleura parietale The femoral vein was cannulated for i infusions. In some of the expt catheters for recording aortic arch and left atrial pressure were introduced through the femoral artery and pulmonary ve n The catheters were flushed with heparin solutio f 5(IU I 100 ml of saline during the course of the pt I m the eft side of the neck the vagosympathetic trunk pre pared in order to record vagal efferentation baroreceptor afferentation and left atrial afferent acti ity Response in vagal efferentation were tested by stimulating electrically the cranial end of the baroreceptor nerve Those effer nt fibres of the vagus nerve which showed spontaneous ef ferent activity capable of being increased by aortic b receptor nerve stimulation were selected for the rec r g of cardiovascular efferent vagal activity The acid-base balance of the experimental animal was monitored b taking blood samples anaerobically from the femoral tery The corrections to acid-base balance and blood g state were made by adjusting the ventilated gas mixtur and respirator rate and by giving i v 7.5% sodium bi bonate The pO_2 of arterial blood was adjusted to 1 140 mmHg pCO to 20–40 mmHg and pH to 7 3 The temperature of the animal was measured from th esophagus and temporarily from the left atrium by mea of an electric thermometer and regulated th a el heated operation table Additional doses of α-chloralose were given when needed The urinary bladder was c nulated The i v infusions were performed through the femoral vein and the temperature of the infused saline was equal to the temperature of the left atrial blood of the e perimental animal The sympathetic activity as recorded from the few-fibres preparation of the third inferior ca diac nerve Vagal efferentation was recorded from a fe fibres. The afferent activities in all the experiments were recorded from single fibres. The action potential w filmed from the screen of a cathode ray tube th a f speed of 15 2 cm/s The impulses were calculated v ually from the film. The number of impulses per second one heart cycle was calculated and the values for stati cal analyses were the mean of the impulses/s from succ sive periods of 4 s The significance of the changes heart rate and nerve activities were studied by a Student *t*-test The correlations between two parameters w tested with regression analysis The changes were s nificant if *P* was less than 0 05 All the calculated valu were determined from a period before (control), duri and after the infusion and from a 10 s period after a 2 break in the recording following the infusion period

RESULTS

In all the experiments attempts were made to r cord simultaneously as many different nerve fibr as possible usually from ? to 4 different nerves In 32 dogs with 42 infusions the discharge rates of aortic baroreceptors 1 left atrial B-type recept 31 sympathetic efferent nerves and 31 vagal cardiac efferent nerves were studied

Heart rate resp nses t infusion

The 4? i v infusion of 80–150 ml of body war saline in 3? dogs within 40–80 s induced tachycardia in 31 cases and bradycardia in 11 cases The control values of heart rate in tachycardic responses varied from 54 beats/min to 158 beats/min mean 91 beats min and th maximal effect during or after infusion wa fr m 8 be ts/min to 185 beats/min mean 1 beat /min The difference in heart rate between the m f control and the mean of the tachycard

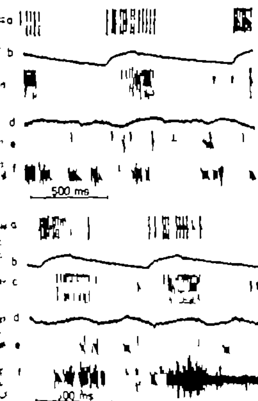


Fig. 1. Influence of infusion upon nerv activities before (A) and 20 after (B) infusion of 170 ml of saline. The traces from top to bottom. (a) Aortic baroreceptor activity, (b) aortic arch pressure, (c) left atrial B-type activity, (d) left atrial pressure, (e) vagal efferent cardiac activity and (f) sympathetic efferent cardiac activity. The weight of the animal was 10.2 kg and the response to infusion tachycardia.

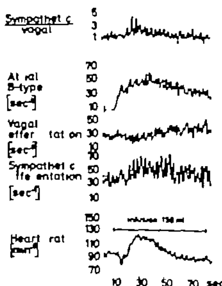


Fig. 2. Influence of saline infusion on the ratio of the number of sympathetic and vagal imp/s, number of left atrial B-type imp/s, number of vagal cardiac efferent imp/s, number of sympathetic postganglionic efferent cardiac imp/ and on the heart rate where the response to infusion is tachycardia. Note the decrease in heart rate at the beginning of the infusion. The values represent results calculated from cycle to cycle. The weight of the animal was 12.7 kg.

response was highly significant ($P<0.001$). In 10 out of 31 infusions the beginning of the infusion induced decrease in heart rate during the first 4–10 heart beat which was followed by tachycardia, cf. Fig. 2. In bradycardic responses the control heart rate values varied from 101 beats/min to 169 beats/min, mean 122 beats/min and during the infusion from 78 beats/min to 153 beats/min, mean 97 beats/min. The difference between the mean values of the two groups was highly significant ($P<0.001$).

Nerve activities in tachycardia

In tachycardic responses with 31 infusion the sympathetic efferentation increased in 24 (78%), decreased in 1 (3%) ($P<0.05$–0.001) and was not changed in 6 (19%) ($P>0.05$) of the infusions. Vagal efferentation increased in 4 (13%), decreased in 20 (64%) and no changes were observed in 7 (23%) of the cases. The ratio of sympathetic to vagal impulses increased in 26 (83%), decreased in 0 (0%) and was not significantly changed in 5 (17%) of the cases. Aortic baroreceptor activity rate increased significantly in all the tachycardic responses as did the left atrial B-type activity rate. Typical changes in afferentations and efferentations are demonstrated in Figs. 1 and 2. The correlation coefficients (r) between the heart rate and the sympathetic efferentation varied from 0.144 to 0.917, mean of 0.702, $n=18$, $P<0.001$ of mean value and from -0.226 to -0.865, mean of -0.577, $n=1$, $P<0.01$ of mean value. The correlation coefficients between heart rate and vagal efferentation varied from -0.377 to -0.983, mean of -0.678, $n=18$, $P<0.001$ of mean value and from 0.275 to 0.799, mean of 0.478, $n=19$, $P<0.001$ of mean value and

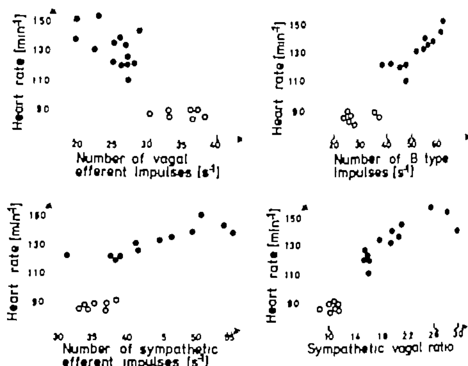


Fig 3 Heart rate as a function of the number of vagal cardiac efferent imp/s ($r=-0$ 891 upper left) number of left atrial B-type imp/s ($r=0$ 950 upper right) number of sympathetic postganglionic efferent cardiac imp/s ($r=0$ 866 lower left) and as the ratio of the numbers of sympathetic and vagal cardiac efferent imp/s ($r=0$ 887 lower right) Each point represents a mean of the results calculated in respect to successive periods of 4 s before (open circles) during and after (closed circles) infusion of saline of 80 ml within 90 s The weight of the animal was 10 6 kg and the response tachycardia.

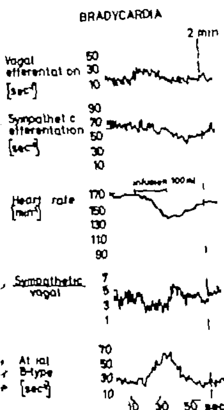
the correlation coefficient between the heart rate and sympathetic vagal ratio varied from 0 222 to 0 937 mean of r 0 691 $n=19$ $P<0$ 001 of mean value The correlation between the B type impulses and heart rate was from 0.546 to 0 976 mean of r 0 828 $n=19$ $P<0$ 001 of mean value Fig 3 represents a typical example in correlations between the heart rate and calculated nervous parameters in tachycardic responses

Nerve activities in bradycardia

In bradycardic responses with 11 infusions sympathetic efferentation increased in 0 (0%) decreased in 8 (75%) ($P<0$ 05–0 001) and was not changed ($P>0$ 05) in 3 (25%) of the cases Vagal efferentation increased in 3 (27%) decreased in 2 (18%) and was not changed in 6 (55%) of the cases The ratio of sympathetic to vagal impulses increased in 3 (28%) decreased in 4 (36%) and was not changed in 4 (36%) The aortic baroreceptor and left atrial B type activity rate increased in all of the cases In bradycardic responses the correlation coefficient (r) between the heart rate and sympathetic efferentation varied from 0 199 to 0 914 mean of r 0 6[illegible] $n=18$ $P<0$ 01 of mean value and between heart rate and vagal efferentation from -0.360 to 0 [illegible] mean of r -0 466 $n=18$ $P<0$ 05 of mean value [illegible] from 0 364 to 0 727 mean of r 0 459 [illegible] $P<0$ 05 of mean value The correlation coefficient between the heart rate and the sympathetic vagal ratio varied from 0 20? to 0 912 mean of r [illegible] $n=17$ $P<0$ 05 of mean value and from -0.4[illegible] -0 737 mean of r -0 605 $n=21$ $P<0$ 01 of mean value The correlation coefficients between B-type impulses and the heart rate varied from -0.11[illegible] -0 885 mean of r -0 430 $n=17$ $P<0$ 1 of [illegible] value Fig 4 represents changes in vagal and sympathetic efferentation heart rate the sympathetic vagal ratio and left atrial B-type activity in a bradycardic response to i v saline infusion.

DISCUSSION

In this study attempts were made to record simultaneously the changes in left atrial aortic baroreceptor and sympathetic and vagal efferent ac



4 Influence of saline infusion upon the number of vagal cardiac efferent imp/s, number of sympathetic postganglionic efferent cardiac imp/s the heart rate the ratio number of sympathetic to vagal imp/s and upon the number of left atrial B type imp/s The response to infusion is bradycardia The values represent result calculated from cycle to cycle The weight of the animal was 13 kg

vities and to find out what is their role separately and together in heart rate regulation One of the difficulties met in this work was to find and get most of the four different types of nerves studied functioning together and simultaneously For this reason the recording of aortic and atrial pressures with atrial and baroreceptor activities was done only in a few expt The use of heparin addition to the use of cannulae caused an increased tendency to bleed in nerve preparations It is clear that single and few fibre recordings from sympathetic and vagal nerves are a practical way of studying afferentation and efferentation but it is also obvious that they represent only a small part of all the information transfer between the central nervous system and the heart However changes in the activity of these fibres would give some new information on their role in the phenomenon studied

The morphine premedication may augment the vagal tone (Page & Hoff 1969) which of course has a favourable effect on eliciting the tachycardic reflex Anesthesia with α-chloralose was chosen, because it leaves the autonomic balance and reflexes largely intact (Price 1960, Greisheimer 1965 Cox 1972) Pentobarbital was not considered to be proper because it has been proved to depress the cardiovascular reflexes and nervous activities (Hallinen Hakumäki & Sarajas 1978) in spite of the fact that it has been widely used in cardiovascular research physiology

The main reaction with 42 i.v infusions of saline in 32 dogs was tachycardic In 10 infusions of these a decrease in heart rate at the beginning of the infusion during the first 4–10 heart beats was found. It is possible that these temporary heart rate changes are merely due to the augmented mechanical load on the heart induced by increased venous return at the beginning of the infusion. However it seems that also constant bradycardia is one of the responses to i. infusion in anesthetized dogs in addition to tachycardia. The mean of the control heart rate in tachycardia was significantly lower than the mean of the control heart rate in bradycardia, but also the bradycardic responses were observed with a low control heart rate

The findings about efferentation in tachycardia in the present work are in agreement with the earlier works of Bergstrom Hakumäki & Sarajas (1971) Hakumaki (1972) that sympathetic efferent cardiac activity increases in tachycardic responses. Also opposite reactions were observed in the present work, which implies that there might be more specific reflex arches so that different cardiac nerves might be activated separately and independently

Vagal efferentation showed diminution in most of the cases in tachycardia, although increases in activity were also observed. It is poss ble that all the nervous activity recorded as cardiac efferent impulses might ha e contained impulses of efferent fibres to some other organs which increased their activity by cardiovascular stimulation. It is clear that vagal efferentation alone does not regulate heart rate but the sympathetic-vagal ratio seem to be one of the most effective regulators in tachycardic responses. This ratio was increased in 83% of the cases, a decrease was not observed. The re

sults of efferentation in this work support the hypothesis of a reciprocal function of the sympathetic and vagal cardiac efferentation reported earlier by Marguth Raule & Schaefer (1951) and later Weidinger Hetzel & Schaefer (1967) However this antagonism seems not to be the rule because different types of reactions were also seen in vagal and sympathetic cardiac activity It is obvious that the heart rate is a result of the sum function of the sympathetic and vagal activities and the ratio of sympathetic to vagal impulses as a main regulator allows different types of reactions in both the autonomic nervous activities

In bradycardic results the findings in efferentation are quite opposite to the findings during tachycardia In most cases a very clear decrease in sympathetic cardiac efferent activity was observed although there were some nonsignificant changes There was never an increase in activity A very important point in these results is that in vagal efferentation activity was not greatly increased and in half of the cases was not changed significantly It is also confusing that the sympathetic vagal ratio does not correlate clearly to the heart rate in bradycardia It is possible that bradycardia during volume load is a result of a combination of cardiac contractility changes and neural factors So the bradycardic response can be explained in the following way The pumping capacity of the heart has decreased in the course of the experiment and by i v infusion the increased volume load thus stretches the weakened ventricles and stimulates the ventricular receptors more than usual This overwhelms the tachycardia initiating neural systems and decreases the sympathetic efferentation to the heart This hypothesis supports the findings of this work that 10 tachycardic reactions were preceded by a short period of 5–10 heart beats of bradycardia at the beginning of the infusions Also the recent observations of Wennergren Thorén & Lisander (1977) support this hypothesis of the role of the ventricular receptors in bradycardia

What is the role of the left atrial B-type receptor and aortic arch baroreceptor activities in these results? In all the tachycardic and bradycardic responses the activity rates of these receptors increased The left atrial B-type receptor has both an inhibitory (Hakumäki 1970) and excitatory influence on sympathetic efferentation (Kappagoda et al 1975 Koizumi et al 1975) so that initial response to left atrial stretch is inhibitory and the later responses excitatory in sympathetic cardiac efferent activity It is obvious that the increased activity of atrial receptors is responsible for the increase of cardiac sympathetic efferentation and heart rate It is difficult to explain why atrial receptors do not influence the sympathetic system in bradycardic responses though they increased their activity The aortic arch baroreceptor may inhibit atrial receptor influence in CNS in bradycardia but why they do not do it in tachycardic responses where the baroreceptors also increased their activity is unclear It may be concluded that baroreceptors do not play a significant role in heart rate regulation during volume load as Bishop & Peterson (1976) have earlier suggested in conscious dogs It is possible that a more detailed analysis of the baro- and atrial receptors would give new information on their role in heart rate regulation at the CNS level

It is interesting that most of the observations and conclusions made in this field on neural cardiovascular regulation have been correct or partly correct It can be assumed that the so-called Bainbridge reflex is a sum function of different kinds of overlapping inhibitory and excitatory components which are working simultaneously during and after the i v infusion and the responses in the heart rate are dependent upon that reflex arch which is stronger and overwhelming the others. It seems that there are still numerous open questions in the nervous control of heart afferentation and efferentation and in their interrelationships in different kinds of cardiovascular conditions and more experimental work is needed with anesthetized and conscious animals before the details of cardiovascular regulation are well-known

This work was supported by the Academy of Finland, the North-Savo Regional Fund of the Finnish Cultural Foundation and the Paavo Nurmi Foundation. I wish to express my gratitude to Mrs Raija Holopainen and Mrs Eija Holma for typing the manuscript and drawing the figures.

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a Physiol Scand 1979 107 135–139

n the chromaffin system of the African lungfish *otopterus aethiopicus*[1]

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ABRAHAMSSON T HOLMGREN S. NILSSON S & PETTERSSON K. On the chromaffin ystem of the African lungfish, Protopterus aethiopicus. Acta Physiol Scand 1979 107 135–139 Received 5 March 1979 ISSN 0001-6772. Department of Zoophysiology University of Göteborg, Sweden.

The distribution of chromaffin tissue was studied in the African lungfish using Falck Hillarp fluorescent histochemistry together with quantitative analysis of catecholamines in plasma and tissue extracts. Intensely fluorescent cells form chromaffin tissue in the wall of the atrium, the wall of the most anterior part of the left cardinal vein and the walls of the segmentally arranged intercostal arteries. The arrangement thus appears to be combination of the situations in cyclostomes elasmobranchs and teleosts. Adrenaline is present in larger quantities than noradrenaline in the intercostal arteries and the cardinal vein, while noradrenaline dominates in the atrium During stress induced by physical disturbance of the animals strong increase in especially the noradrenaline concentration of the plasma was detected

Key ords Lungfish, *Protopterus aethiopicus* chromaffin tissue, catecholamines

n 1900, Kohn introduced the term chromaffin for ells which are stained brown by certain chrome alts, a phenomenon first described by Henle (1865) n experiments with the adrenal gland. Coupland 1965 1977) defines a normal chromaffin cell as one which develops from neuroectoderm is innervated by pre-ganglionic sympathetic nerve fibres, is capable of synthesizing and secreting catecholamines and of storing them in sufficient concentration to give a positive chromaffin reaction

In studies of chromaffin ystems of lower vertebrates it has rarely been possible to obtain enough evidence to fulfil all these criteria, and the term chromaffin is therefore used in less exact sense to indicate cells storing large quantities of catecholamines

Well developed chromaffin systems are found in a number of lower vertebrates, but contrary to mammals the chromaffin tissue is not arranged a a distinct adrenal medulla. I cyclostomes (hagfish and lamprey), chromaffin tissue has been described in the walls of major veins (Giacomini 1902, Gaskell 1912) and there are also large quantities of catecholamines stored in chromaffin cells of the heart (both ventricle and atrium) and in the portal vein heart of Myxine (Östlund 1954 Augustinsson et al 1956, Östlund et al. 1960 Euler & Fänge 1961 Bloom et al 1961 Lagerstrand & Nilsson 1972) Elasmobranchs have their chromaffin tissue arranged segmentally along the vertebral column into chromaffin bodies. These are sometimes referred to as supra-renal" bodies as opposed to the inter renal" tissue which is the adreno-cortical tissue of the elasmobranchs. The supra-renal" bodies of the elasmobranchs are associated with sympathetic ganglia and may be innervated by pre-ganglionic fibres of sympathetic origin. Especially large chromaffin bodies lie in the anterior part of the cardinal sinus in contact with the gastric ganglion on each side ("axillary bodies") (Young 1933 Nicol 1952 Coupland 1965 Gannon et al. 1972 Nilsson et al 1975 Abrahamsson 1979)

In teleosts the chromaffin tissue is embedded in

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[1] Part of this work as presented at the meeting of the Scandinavian Physiological Society in Lund. Acta Physiol Scand 1978, 102: 33A–34A.

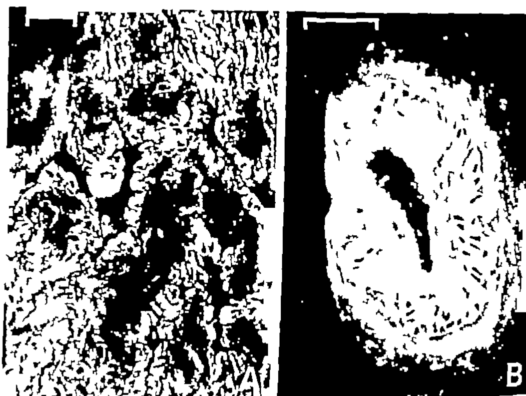


Fig 1 (A) Intensely fluorescent cells lining the lumen of the atrium Calibration = 100 μm. (B) Intensely fluoresc cells in the wall of an intercostal artery very close to the dorsal aorta Calibration = 100 μm

the walls of the posterior cardinal veins inside the head kidney (Oguri 1960 Mazeaud 197? Grove et al 1972 Nilsson 1976) The chromaffin tissue in the head kidney of the cod is innervated by pre-ganglionic cholinergic fibres which control release of adrenaline and noradrenaline into the blood stream (Nilsson et al 1976 Holmgren 1977)

Chromaffin cells in the lungfish Protopterus annectens were described by Giacomini (1906) They were found in the walls of the intercostal arteries and also in the anterior part of the left cardinal vein (azygos vein") Holmes (1950) confirmed the finding of chromaffin cells in the intercostal arteries of the same species but was unable to verify Giacomini's finding of such cells in the cardinal vein Sympathetic fibres reach the left cardinal vein and an innervation of the chromaffin tissue in the intercostal arteries has been described (Jenkin 1928 Holmes 1950)

This work was started to clarify the distribution and function of chromaffin cells in a Dipnoan fish by use of Falck Hillarp fluorescence histochemistry and analyses of catecholamine content in the organs which from findings in other lower vertebrates can be suspected to store catecholamines

MATERIAL AND METHODS

African lungfish (Protopterus aethiopicus) of either se with a body length of 35–50 cm were used in the exper ments The animals were captured near Kisumu, Keny and flown from Nairobi to Göteborg where they ere kep in fresh water at 25°C and regularly fed before the exper ments.

Measurement of catecholamine levels in plasma an tissues were made by the method of Bertler et al (1958) modified by Häggendal (1963) The catecholamines perchloric acid extracts of plasma and tissues er purified on Dowex 50W X8 resin and fluorometric mea urement were made using a Farrand Mark I spectrofluorimeter The recovery of known amount adrenaline (100 ng) and noradrenaline (100 ng) from the columns was determined and found to be adrenaline 82± % (mean ± S E. =11) and noradrenaline 83± (mean ± S E. *n* = 11) The values given below have be corrected for these recoveries All values are presented means ± S E Stress was induced by chasing the anim around its tank for 10–15 min Blood samples ere take with a heparinized syringe from the dorsal aorta im mediately afte killing

Fluorescent histochemistry wa carried out by the F lck Hillarp method as described by Falck & Owma (1965) Freeze-dried pieces of tissue were treated formaldehyde vapour at 80°C for 1 or 3 h Tissue section of 10 μm were mounted i Entellan (Merck) and viewed a Leitz Orbolux fluorescence microscope with top lig illumination and barrier filter Photograph

Pi sed 3 and 10

le 1 Catecholamine level in some tissues and plasma of the African lungfish expressed as µg/g tissue µg/100 ml plasma spect ely

alues are given as means ± S.E. Number of estimations =

ue	Adrenaline	Noradrenaline	% adrenaline	
rt	4 18±1 10	70.77±17.31	5	6
sal aorta proximal part of tercostal arteries	5 10±2 06	0.26±0 4	98	5
ximal part of intercostal teries	216	94.3	70	1
ercostal arteries distal portion	0.33±0 08	0 05±0 03	87	8
rdinal vein (left)	0.55±0 23	0.03±0 02	95	6
od plasma (control)	2.29±0 77	2.36±1 15	49	6
od plasma ("stressed")	6 41±1.85	29 10±14 28	18	4

re taken by Leitz Orthomat automatic camera on dak Tri-X film

SULTS

rge quantities of intensely fluorescent chromaffin lls ere found by the Falck Hillarp fluorescent stochemical technique in the atrium of the heart, most proximal part of the intercostal arteries d in the anterior 5 mm of the left cardinal ein gs 1 and).

In the atrium, the fluorescent cells line all inner urfaces of the pongy tissue (F g 1 A) No such ells were present in the ventricle A similar lining f the essel wall by fluorescent cells was observed n the left cardinal ein (Fig), while in the inter ostal arteries there appeared to be layer of non-fluorescent tissue between the chromaffin tissue and the essel lumen (Fig. 1 B) It was not poss ble to detect any differences in the intensity related to ormaldehyde incubation times (1 or 3 h) Table 1 hows the result of the catecholamine analysis of he different tissues and plasma It is notable that noradrenaline dominates over adrenaline in the heart and also in the plasma of the stressed" animals hile adrenaline appears to dominat in the other chromaffin tissues I one animal the exact part of the intercostal arteries containing the chromaffin tissue was carefully d sected out giv ing higher catecholamine content per g tissue (Table 1)

DISCUSSION

Chromaffin tissue and sympathetic fibres innervating this tissue was described by Giacomini (1906) in the intercostal arteries of Protopterus annectens and later by Holmes (1950) The present findings of the position of the chromaffin tissue in the inter costal arteries of P aethiopicus a species closely related to P annectens agree well with these earlier descriptions. A further claim by Giacomini (1906) that chromaffin cells were also present in the left cardinal vein was refuted by Holmes (1950) on the grounds that the chromaffin reaction used was not specific enough to allow such a statement. From the present work, where the highly specific fluorescent histochemical technique was used it is evident that Giacomini was right, and that there is a well de veloped chromaffin tissue in the anterior part of the left cardinal vein ("azygos vein")

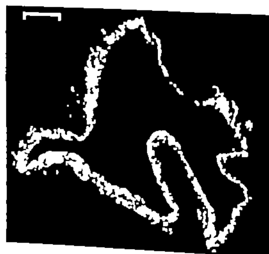


Fig 2 Intensely fluorescent cells lining in the walls of the most anterior part of the left cardinal vein. Photomontage Calibration = 200 µm.

In addition to confirmation of earlier findings large quantities of chromaffin cells were found in the atrium of the heart This is similar to the situation in cyclostomes where adrenaline and noradrenaline in large amounts have been found in both atrium and ventricle (Lampetra Petromyzon Myxine) and also in the portal vein heart (Myxine) (Östlund 1954 Lagerstrand & Nilsson 1972)

Thus it is clear that Protopterus has a remarkably well developed chromaffin system with its chromaffin tissue organized as a "combination" of the systems seen in several of the other groups of lower vertebrates

Although the main catecholamine of the chromaffin tissue in the intercostal arteries and the left cardinal vein appears to be adrenaline noradrenaline is the circulating amine showing the most dramatic increase during "stress This amine is stored in large quantities in the atrium and it is possible that a large portion of the circulating catecholamines are released from the heart An innervation of chromaffin tissue in the intercostal arteries and left cardinal vein has been described (Jenkin 1928 Holmes 1950) but the nature of the control of catecholamine release from the atrium is not known

The estimation of the catecholamine content of the heart must be evaluated considering the fact that only the atrium contains fluorescent cells The amount of amines per gram tissue present in the atrium is therefore more than twice the figure given in Table I Similarly the weight of chromaffin tissue proper embedded in the vessels (intercostal arteries/dorsal aorta and cardinal vein) is very small compared to the total weight of the vessel tissues and therefore the amounts of amines per gram chromaffin tissue are much higher than indicated by the presented figures This is also shown by the much higher catecholamine content in the sample of carefully dissected intercostal arteries (Table I)

The strong increase in the concentration of especially noradrenaline in blood plasma following physical disturbance ("stress) indicates that circulating amines play a role in the over-all control of circulation and other organ functions The effects of circulating amines may be of particular importance in *Protopterus* since the sympathetic adrenergic system is very poorly developed (Abrahamsson et al 1979*a*)

It is concluded from the present study that the chromaffin system of the African lungfish is composed of chromaffin tissue in at least three different regions the walls of the intercostal arteries the wall of the left cardinal vein and the wall of the atrium Adrenaline is present in larger quantities than noradrenaline in the intercostal arteries and the left cardinal vein while in the atrium—and also in the blood plasma of "stressed" animals—there is a dominance for noradrenaline Preliminary results indicate that the noradrenaline in the Protopterus heart can be synthesized locally and not only taken up and stored as suggested in certain teleosts (Saetersdal et al 1974 Abrahamsson et al 1979b

Our thanks go to Mrs M Fernandes and Prof M Hyder Zoology Department University of Nairobi, for their help in obtaining the animals We also sincerely thank B Egnér and Mrs G Rydgren for their expert technical assistance This work was supported by grants from the Swedish Natural Science Research Council the Magn Bergvall Foundation and the Lars Hierta Memorial Foundation

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drenergic and cholinergic effects on the heart ıe lung and the spleen of the African lungfish *rotopterus aethiopicus*

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ABRAHAMSSON T HOLMGREN S NILSSON S. & PETTERSSON K. Adrenergic and cholinergic effects on the heart, the lung and the spleen of the African lungfish Protopterus aethiopicus Acta Physiol Scand 1979 107 141 147 Received 5 March 1979 ISSN 0001-6772 Department of Zoophysiology University of Göteborg, Sweden

Effects of adrenergic and cholinergic drugs were tudied on isolated preparations from the heart, the lung and the spleen of the African lungfish In addition, nerve-lung preparation was employed for the study of the autonomic nervous control of the lung. Falck Hillarp fluorescent histochemistry was used in the search for adrenergic neurons in the sympathet ic chain and tissues. The Protopterus lung received cholinergic excitatory innervation via the pulmonary branches of the vagi. The presence of parasympathetic ganglia in the lung tissue is indicated Adrenergic drugs, when producing response either contracted or relaxed lung strips Spleen strips were contracted by cholinergic drugs acting on muscarinic receptors and, to lesser extent, by adrenergic drugs A strong negative inotropic effect was produced by carbachol on paced atrial but not ventricular strips, while adrenaline increased the contraction force in ventricular but not atrial strips. The sympathetic ganglion cell did not contain enough catecholamines to give off any visible fluorescence, nor as it possible to show any adrenergic nerve terminals in the tissues studied. It is concluded that the adrenergic control in the lungfish is mainly due to circulating catecholamines, rather than adrenergic nerve fibres, since concentrations of catecholamines in Protopterus plasma are high enough to affect the spleen, lung and ventricle

Key ords Lungfish Protopterus aethiopicus, drug effects, autonomic nervous ystem

The anatomy of the ympathetic nervous system in lungfish was first described by Giacomini (1906) (Protopterus annectens) and in more detail by Jenkin (1928) using another pecies of the family Lepidosirenidae (Lepidosiren paradoxa) The ympathetic chains in both animals are extremely fine chords extending from the posterior part of the body cavity and ending anteriorly (in Lepidosiren) at the 1st spinal nerve Segmental sympathetic ganglia lie in close connection with the intercostal arteries and ympathetic innervation of the chromaffin c lls in the wall of these arteries has been described (Holmes 1950 Protopterus annectens) A ympathetic branch to the left cardinal ein (Carygos ein), which may also control chromaffin tissue in this ein has also been described (Jenkin 1928)

There are no direct connections between the sympathetic chains and the vagi but sympathetic fibres might at least theoretically reach the vagi via the nerve join ng the vagus ganglion and the 1st pinal nerve (Jenkin 1928)

The lung is innervated by autonomic nerve fibres running in the pulmonary branches of the vagi the left pulmonary vagus innervates the right part of the lung and vice versa. Ganglion cells in the pulmonary vagi has been described (Parker 1892, Giacomini 1906 Jenkin 1928) Acetylchol ne contracts and catecholamines relax the Protopterus lung, the effect of acetylcholine being blocked by atropine (Johansen & Reite 1967).

The heart does not appear to be under a cholinergic vagal tonus since atropine does not change the heart rate while high doses of

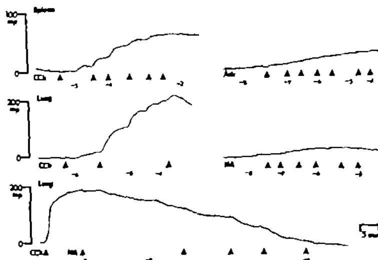


Fig 1 Recordings of drug-induced tension changes in isolated strip preparations from the spleen (upper traces) and lung (middle and lower traces) of Protopterus. Numbers refer to the logarithm for the agonist concentration in mol/litre (*M*). The following agonists were used adrenaline (*Adr*), noradrenaline (*NA*) and carbachol (*CCh*). Note that the preparation represented by the lower trace was precontracted by carbachol (10^{-4} M)

Adrenaline or noradrenaline either contracted (pD$_2$ for adrenaline 5 89±0 97 =3) or after pre-contraction with carbachol, relaxed (pD$_2$ for adrenaline 5 48 5.05 =2) the lung strips (Fig. 1) Low concentrations (10^{-10} 10^{-8} M) of the beta adrenergic agonist isoprenaline relaxed precontracted lung strips, while high concentrations (10^{-6} 10^{4} M) produced contraction of previously untreated strips. Similar to the spleen strips lung strips showed a much lower sensitivity of adrenergic drugs compared to cholinergic drugs tested on the same preparation, and in 4 fish no recordable response at all to adrenergic drugs was obtained.

A strong negative inotropic effect was caused by carbachol on *atrial strip* from one fish tested, while the effect on *ventricular strip* was small or absent 4) (Fig 2) Positive inotropic responses were obtained with adrenaline on ventricular (pD$_2$= 6 69 0 17 maximal increase in beat force 30 0 1 0 n 3) but not atrial (n = 1) strips (Fig.)

Isolated nerve-lung preparation

Contractions of the lung could be obtained by electrical stimulation of the pulmonary branch of the vagus every 2 min for at least 6 h. Preparations from 2 animals were used. The force of contraction and the duration of each contraction could be increased by addition of eserine (10^{-6} M) to the organ bath (Fig 3) Addition of *d*-tubocurarine (7 6×10^{-6} M) or atropine (10^{-6} M) strongly diminished or abolished the responses to nerve stimulation (Fig. 3)



Fig 2 Recordings of inotropic responses of Protopterus ventricle (upper traces) and atrium (lower traces) to adrenaline (*Adr*) and carbachol (*CCh*). Numbers refer to the logarithm for the agonist concentration in mol/litre (*M*) The isolated strip preparations were stimulated with pulses of 10 V and 1 ms duration at 0 8 Hz.

acetylcholine produces bradycardia (Johansen & Reite 1968) Positive ino- and chronotropic effects of adrenaline have been recorded the chronotropic effect being most pronounced in aestivating animals (Mohsen et al 1974)

The spleen is an elongated organ embedded in the gut wall and is rich in lymphoid tissue (Parker 1892) No direct sympathetic innervation of the type seen in other vertebrates (splanchnic nerves) appears to be present in lungfish (Jenkin 1928) and if any sympathetic fibres at all reach the spleen they must run with the intestinal branches of the vagus

This work aims to elucidate the significance of the autonomic nervous system in the African lung fish and the effects of cholinergic and adrenergic drugs on the lung the heart and the spleen

MATERIALS AND METHODS

African lungfish Protopterus aethiopicus of either sex with a body length of 35–50 cm were used in the experiments The animals were captured near Kisumu Kenya, and flown from Nairobi to Göteborg where they were kept in fresh water at 25°C and regularly fed before the experiments

Isolated strip preparations

Strips with a thickness of 3 mm were cut from the atrium and ventricle of the heart the spleen and the lung of freshly killed lungfish The strips were attached to GRASS FTO3 isometric transducers and suspended in organ baths containing a lungfish ringer of the following composition. NaCl 5.5 KCl 0 33 $CaCl_2$ $2H_2O$ 0 32 MgSO $7H_2O$ 0 19 NaHCO 2 0 NaH_2PO $2H_2O$ 0 43 glucose 1 0 g/litre The ringer was kept at 25°C and bubbled throughout the experiment with 97% O_2/3% CO_2 Strips kept in ringer at +4°C could be used for up to 4 days without detectable changes in responses A basic tonus of 25 mp (1 mp = vertical force of 1 mg = 9 81×10⁻⁶ N) was initially appl ed to the strips which were then left to recover for 1 h before the start of experiments.

The atrial and ventricular strips were paced by electrical pulses of 0 8–1 Hz 1 ms and 7–10 V delivered from GRASS S6 stimulators through two thin platinum wires attached around the lower parts of the suspended strip

Concentration/response curves were obtained by cumulative addition of drugs and affinity for the drugs determined as pD_2-values (pD = −log EC_{50}) (van Rossum 1963) pD_2 values are given±S E M and *n* indicates number of animals From each animal 1–6 preparations were used for pD determinations and mean values used for further determinations The pA_2/pA_{10}-values for atropine on spleen strips were determined with carbachol as agonist as described by Ariens & van Rossum (1957) and van Rossum (1963)

Isolated nerve–lung preparation

2 3 cm of the pulmonary branch of the vagus were carefully dissected free from the underlying lung lobe and a 1 cm wide ring of the lung was cut posterior to the nerve segment The nerve/lung preparation was suspended in an organ bath as described for the isolated strip preparations and the nerve placed over two fine platinum hooks and regularly stimulated for 20 s with 20 Hz 1 ms duration and 10 V with min intervals

Fluorescent histochemistry

Pieces of the dorsal aorta spleen, lung and sympathetic chain were quick-frozen in liquid propane cooled in liquid nitrogen After freeze-drying, the tissue pieces were treated as described by Falck & Owman (19 Paraformaldehyde treatment at 80°C was carried out for 1 or 3 h Sections of 10 μm were viewed in a Leitz Orthol microscope with top light illuminator and a barrier filter 470 nm With this treatment the presence of catecholamines in the preparations can be identified by a blue-green ("specific") fluorescence

Drugs

The following drugs were used in the experiments acetylcholine chloride L-adrenaline bitartrate atropine sulphate carbachol chloride eserine salicylate isoprenaline hydrochloride L-noradrenaline bitartrate *d*-tubocurarine chloride Concentrations are expressed as mol/litre (M)

RESULTS

Isolated strip preparations

Spleen strips contracted in response to the cholinergic agonists carbachol (pD_2 = 4 59±0 1 *n* = 6) and acetylcholine (pD_2 = 4 09±0 14 *n* = 3) and the adrenergic agonist adrenaline (pD_2 = 6 36±0 *n* = 10) (Fig 1) Noradrenaline produced very weak or not recordable responses not allowing any quantified estimations (*n* = 6) The effects of catecholamines were invariably weaker than the effects of the cholinergic agonists when tested on the same preparations Atropine (10 −10 M) caused a parallel shift of the concentration/response curve for carbachol giving pA_2 = 8.2 and pA_{10} = 9 4 estimated from 7 antagonized curves from 3 fish which indicates a competitive blockade of muscarinic receptors

Lung strips were contracted by acetylcholine (*n* = 2 pD_2 4 96 4 14) and carbachol (pD_2 5 38±0 11 *n* = 8) (Fig. 1) The effects of both acetylcholine and carbachol were antagonized by atropine (10 −10⁻⁶ M) but no evaluation of pA values was made since consecutive control curves with the agonists showed irregular depression of the sensitivity of the strips making absolute determinations of the shift produced by the antagonist impossible

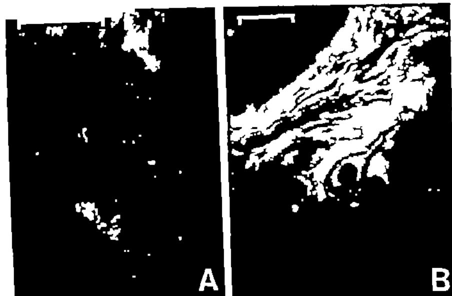


Fig. 4. Sympathetic ganglion cells from Protopterus, detectable in the fluorescence microscope only by their weak non-specific "back-ground" fluorescence and weak orange yellow fluorescence emitted by cytoplasmic granules. The outlines of the cells are clearly visible. In *B* elongated cells emitting an intense specific fluorescence can be seen alongside the nerve cell bodies. Calibration in both *A* and *B* = 50 µm.

The results obtained with atropine and eserine on the nerve-lung preparations are compatible with the view that the pulmonary branches of the vagi carries cholinergic excitatory fibres to the lung. The blocking effect of *d*-tubocurarine, which serves as a good ganglionic blocker in toad (Nilsson 1978) could indicate the presence of autonomic ganglia in the lung. However, no tests to elucidate the selectivity of the ganglionic blockade were performed in this study. The effect of eserine suggests the presence of cholinesterases in the preparation.

An antagonistic adrenergic inhibitory control of the lung could be exerted by circulating catecholamines. The plasma concentrations of adrenaline increases from 0.13 µM in control animal to 0.33 µM in "stressed" animals, while the corresponding figures for noradrenaline are 0.14 µM and 1.7? µM respectively (Abrahamsson et al 1979). These concentrations of the catecholamines will influence the lung musculature as judged from the concentration/response curves for adrenaline and noradrenaline on isolated lung strips.

No studies of chronotropic effects of drugs on the heart were performed in the present work, but positive chronotropic effect of adrenaline on the heart of Protopterus annectens, particularly aestivating animals, have been described (Mohsen et al 1974). Positive inotropic effects of catecholamines on isolated ventricular strips were seen in the present study. Again it is possible that the levels of circulating catecholamines affect the heart in a manner similar to the situation in elasmobranchs and teleosts (Gannon et al 1972, Wahlqvist & Nilsson 1977, Holmgren 1977, Short et al 1977, Butler et al. 1978).

A lack of a negative inotropic effect of cholinergic agonists on the ventricle is found in other vertebrates (Gannon 1971, Hedberg & Nilsson 1976, Holmgren 1977). Similarly in Protopterus the carbachol effect on the ventricle is absent or very small compared to the effect on the atrium. In a teleost (Holmgren 1977) and a reptile (Hedberg & Nilsson 1976) where the inotropic effects of drugs were studied in the same way as in the present work, a distinct positive inotropic effect of catecholamines was observed in the atrium. The lack of this effect in the isolated strip preparations from the lungfish atrium may in some way be linked to the presence of very large quantities of catecholamines in this organ (Abrahamsson et al 1979). Similarly exogenous catecholamines have no effect on the adrenaline and noradrenaline rich

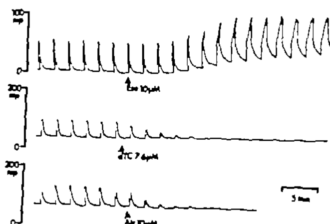


Fig 3 Effects of cholinergic agents on the concentrations of the Protopterus lung produced by electrical stimulation of the pulmonary branch of the vagus nerve with 20 Hz 1 ms duration and 10 V for 20 s every 2 min Addition of eserine (*Ese* 10^{-6} M) produces an increase in contraction force and duration (upper trace) while d tubocurarine (*dTC* 7.6×10^{-6} M) and atropine (*Atr* 10^{-6} M) abolishes the response to nerve stimulation (middle and lower traces)

Fluorescent histochemistry

No fluorescent nerve terminals were observed in any of the organs studied and this may at least in part be due to the presence of strongly autofluorescent material in the tissues However no specific catecholamine fluorescence was observed in the cell bodies of the sympathetic ganglion cells either but only a dull orange yellow granular fluorescence (Fig 4A B) In a few cases cells emitting a very strong specific fluorescence were observed in close proximity to sympathetic ganglion cells (Fig. 4B)

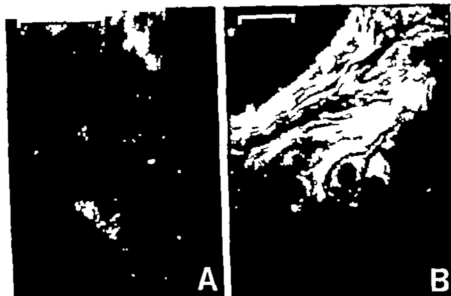
DISCUSSION

The sympathetic ganglia are diffusely arranged in elasmobranchs and chondrosteans while distinct sympathetic chains of the type seen in the tetrapods are present in holosteans and especially teleosts (Nicol 1952) Regularly arranged sympathetic chains are also found in the dipnoans (Giacomini 1906 Jenkin 1928) although early anatomists over looked the very delicate strands (Parker 1892 Wiedersheim 1989 quoted in Jenkin 1928) The present findings that the sympathetic cells do not contain enough catecholamines to produce a visible fluorescence with the Falck Hillarp technique suggests that the sympathetic system of Protopterus plays a very small role as an adrenergic nervous control function The sympathetic system may however be important in the control of catechol amine release from the chromaffin tissue in vario parts of the animal (Giacomini 1906, Jenkin 19 Holmes 1950 Abrahamsson et al 1979). The la of a prominent sympathetic adrenergic mech may in part be compensated for by a more pr adrenergic control by circulating catecholamin

The elongated intensely fluorescent cells in t sympathetic ganglia are similar to cells observed other vertebrates and may have some local re ulatory function like the SIF-cells of mamma ganglia (McLean & Burnstock 1966, Nilsson 19 Tosaka & Kobayashi 1977)

The mammalian spleen is contracted by ca cholamines acting via α adrenoceptors of th *splenic smooth musculature but the effects* of acetylcholine are weak and variable (for referenc see Davies & Withrington 1973) The conver adrenergic excitatory effect is also seen in sple strip preparations from lower vertebrates (O dyke & Opdyke 1971 Nilsson et al 19 Elasmobranchs Vairel 1933 Nilsson & Grov 1974 Holmgren & Nilsson 1975 Teleosts, Va 1933 Nilsson 1978 Amphibians) The effects of acetylcholine are inconsistent in elasmobranch weakly excitatory in the toad (Bufo marinus) a very strongly excitatory and consistent in the tel ost (Gadus morhua) where a double sympath adrenergic/cholinergic innervation of the spleen ha been postulated (Nilsson & Grove 1974 Holmgr & Nilsson 1975) It is notable that the Protopteru spleen strips were more sensitive to cholinergi agonists than to the adrenergic drugs. The spleen of Protopterus is somewhat special with its anatomical position embedded in the gut wall and covered w muscular fibres and peritoneum (Parker 1892) In the present experiments this muscle coat was car fully removed to avoid interference from gut muscu lature

Also the lung strip preparations contracted strongly in response to acetylcholine and carbachol while the contractory effects of catecholamines were weak if at all present In precontracted preparations a consistent relaxing effect of catecholamines was observed which is in agre ment with the previous findings of Johansen & Reite (1967) This dual effect of catecholamines might be due to different receptor properties in dif ferent regions of the lung tissue as found in a toad (Bufo marinus) were the lung wall was predomi nantly β-relaxatory while the septal edges were excitatory (Holmgren & Ca 1978)



4 Sympathetic ganglion cells from Protopterus, detectable in the fluorescence microscope only by their weak -specific "back-ground" fluorescence and weak orange-yellow fluorescence emitted by cytoplasmic granules. The ... of the cells are clearly visible. In *B* elongated cells emitting an intense specific fluorescence can be seen alongside nerve cell bodies. Calibration in both *A* and *B* = 50 µm

The results obtained with atropine and eserine on nerve-lung preparations are compatible with the ... that the pulmonary branches of the vagi car... cholinergic excitatory fibres to the lung. The ...cking effect of *d* tubocurarine which serves as a ...od ganglionic blocker in a toad (Nilsson 1978), ...uld indicate the presence of autonomic ganglia ... the lung. However no tests to elucidate the ...fectivity of the ganglionic blockade were per...rmed in this study. The effect of eserine suggest... the presence of cholinesterases in the preparation.

An antagonistic adrenergic inhibitory control ... the lung could be exerted by circulating cate...cholamines. The plasma concentrations of adrena...line increases from 0.13 µM in control animals ... 0.33 µM in "stressed" animals, while the cor...responding figures for noradrenaline are 0.14 ...M and 1.7? µM respectively (Abrahamsson et al. ...979). These concentrations of the catecholamines ...ll influence the lung musculature as judged from ...he concentration/response curves for adrenaline ...nd noradrenaline on isolated lung strips.

No studies of chronotropic effect of drugs on the ...eart were performed in the present work, but posi...tive chronotropic effect of adrenaline on the heart of Protopterus annectens particularly aestivating animals have been described (Mohsen et al. 1974). Positive inotropic effects of catecholamines on isolated ventricular strips were seen in the present study. Again it is possible that the levels of circulating catecholamines affect the heart in a manner similar to the situation in elasmobranchs and teleosts (Gannon et al. 1972, Wahlqvist & Nilsson 1977, Holmgren 1977, Short et al. 1977, Butler et al. 1978).

A lack of a negative inotropic effect of cholinergic agonists on the ventricle is found in other vertebrates (Gannon 1971, Hedberg & Nilsson 1976, Holmgren 1977). Similarly in Protopterus the carbachol effect on the ventricle is absent or very small compared to the effect on the atrium. In a teleost (Holmgren 1977) and a reptile (Hedberg & Nilsson 1976) where the inotropic effects of drugs were studied in the same way as in the present work a distinct positive inotropic effect of catecholamines was observed in the atrium. The lack of this effect in the isolated strip preparations from the lungfish atrium may in some way be linked to the presence of very large quantities of catecholamines in this organ (Abrahamsson et al. 1979). Similarly exogenous catecholamines have no effects on the adrenaline and noradrenaline rich

hearts of cyclostomes (Östlund 1954 Fänge 195? quoted in Östlund 1954) The role of the large quantities of catecholamines in the hearts of cyclostomes and Protopterus in the control of the cardiac work is not known but some local regulatory influence may be suspected

It is concluded from the results of the present work that the sympathetic nervous system plays a minor role as an adrenergic *nervous control* system in the African lungfish although sympathetic fibres may be responsible for an adrenergic *humoral control* by release of catecholamines from stores in chromaffin tissue The sensitivity of the lung spleen and ventricle to catecholamines suggests the possibility of a regulatory function of circulating levels of amines in these organs

Our thanks go to Mrs M Fernandes and Prof M Hyder Zoology Department University of Nairobi for their help in obtaining the animals We also sincerely thank Mrs Lena Utter for her expert technical assistance and angelic patience This work was supported by grants from the Swedish Natural Science Research Council the Magnus Bergvall Foundation and the Anna Ahrenberg Foundation

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techolamine synthesis in the chromaffin tissue ıthe African lungfish *Protopterus aethiopicus*

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ABRAHAMSSON T JONSSON A.-C & NILSSON S Catecholamine ynthesis in the heart and intercostal arteries of the African lungfish Protopterus aethiopicus. Acta Physiol Scand 1979 107 149–151 Received 5 March 1979 ISSN 0001-6772. Department of Zoophysiology University of Goteborg, Sweden

The activities of dopamine-β-hydroxylase (DBH E.C. 1 14.17 1) and phenylethanol-N-methyl transferase (PNMT E.C 2 1 1 10) were determined in tissue homogenates from the chromaffin tissue containing atrium and intercostal arteries from the African lungfish. Clearly measurable activities of DBH were found in both kind of tissues, while PNMT activity was ascertained only in the intercostal arteries. The activity of DBH in the atrium indicates possibility for local synthesis of the large amounts of catecholamines, especially noradrenaline, found in this tissue Catecholamines synthetized in and released from the chromaffin tissues of Protopterus, may be of great importance for the adrenergic control in this animal in the absence of well developed sympathetic nervous ystem.

Ke words Lungfish, Protopterus aethiopicus, catecholamines, dopamine-β-hydroxylase phenylethanolamine-N-methyl transferase

rge quantities of catecholamines are stored in romaffin cell in the systemic and portal vein arts of cyclostomes (Giacomini 1902 Gaskell 91., Östlund 1954 Lagerstrand & Nilsson 197?). storage of catecholamines in endothelial cells of e atrium has been described in two species of eosts (Gadus morhua and Lebistes reticulatus) hese endothelial catecholamines are thought to be aken up from the plasma and stored rather than cally synthetized (Saetersdal et al 1974)

The last tw teps in the biosynthesis of adrenaline in the "Blaschko pathw y (Blaschko 939) are the hydroxylation of dopamine to noradrenaline and the subsequent methylation of noradrenaline to adrenaline The enzymes responsible for hese reactions are dopamine-β-hydroxylase (DBH C 1 14 17 1) and phenylethanolamine N-methyl transferase (PNMT E.C .1 1 10) respectively These enzymes hav been demonstrated in lower ertebrates such s elasmobranchs (Abrahamsson 1979 PNMT), teleost (Abrahamsson & Nilsson 1976 Mareand 1972, Abrahamsson 1979*b* PNMT Jönsson & Nilsson 1979 DBH) and amphibians (Wurtman et al 1968 PNMT Wooten & Saavedra 1974 PNMT and DBH) The properties of DBH and PNMT from lower ertebrates appear to be similar to those of mammalian enzymes with the possible exception of a lower temperature optimum (Wurtman et al 1968, Wooten & Saavedra 1974 Abrahamsson 1979*b* Jonsson & Nilsson 1979)

I a study of the chromaffin system of the African lungfish large quantities of adrenaline (4.2 μg/g whole heart) and especially noradrenaline (70.7 μg/g whole heart) were shown to be present in the atrium (Abrahamsson et al 1979) Chromaffin tissue also lies embedded in the walls of the intercostal arteries, and in the left posterior cardinal vein (Giacomini 1906, Abrahamsson et al. 1979)

This very preliminary work was carried out to investigate the presence of DBH and PNMT in chromaffin tissue from the African lungfish.

MATERIALS AND METHODS

Tissues from two specimens of the African lungfish (Protopterus aethiopicus) were used in this study The animals were captured in Lake Victoria near Kisumu, Kenya, and flown to Göteborg where they were kept in fresh water tanks at 25°C until the experiments.

Table 1

Tissue sample	Tissue weight (mg)	DBH activity (nmol octopamine formed/h×g tissue)	PNMT activity (nmol metanephrine formed/h g tissue)
Intercostal arteries animal 1	10 1	53 8	3_6
Intercostal arteries animal 2	9 4	79 9	117.2
Atrium animal 1	286 4	401 9*	
Atrium animal 2	511_	176 8*	

Mean value from triplicate incubations.

The atria of the hearts or pooled samples of the intercostal arteries were homogenized in 4 and 2 ml 0.14 M KCl respectively The homogenate was then divided into two parts, for the determination of the activities of PNMT and DBH respectively

PNMT was determined by the method of Axelrod (1962) exactly as described by Abrahamsson (1979*b*) Incubation with DL-normetanephrine as substrate was carried out for 30 min at 30°C and pH=7 9 using ¹⁴C S-adenosylmethionin as the methyl donor

DBH was determined essentially according to the method of Nagatsu & Udenfriend (1972) as described by Jönsson & Nilsson (1979) One drop of Triton X 100 was added to the homogenate and 0 4 ml samples were incubated for h at 30°C and pH=5 4 with tyramine as the substrate

RESULTS AND DISCUSSION

No optimation of the various incubation parameters were performed on this very small material The determinations of enzymatic activities were made under conditions shown to be optimal for the respective enzyme from other fish (Abrahamsson & Nilsson 1976 Abrahamsson 1979*a* *b* Jönsson & Nilsson 1979) giving the results presented in Table 1 The exact determination of the enzymatic activities for comparison with other chromaffin tissues is also made difficult by the great variability in the amounts of non-chromaffin vascular and other tissues homogenized together with the small amounts of chromaffin tissue proper It is however evident from the present preliminary results that both PNMT and DBH are present in the intercostal arteries and that DBH in addition is present in the atrium of the heart The possible presence of PNMT in the atrium cannot be ascertained in the present study due to very high blank values These high blank values presumably arise due to conversion of endogenous catecholamines to ¹⁴C methylated metabolites The activity of PNMT in the atrium can however be expected to be low since noradrenaline greatly dominates over adrenaline in this tissue (Abrahamsson et al 1979*a*).

The role of catecholamines thus locally synthetized in the atrium is not known, but a local regulatory function may be speculated The chromaffin tissue of Protopterus may also be of great importance in the general adrenergic function of this animal since a sympathetic adrenergic nervous system appears to be lacking (Abrahamsson et al 1979...

Our thanks go to Mrs M Fernandes and Prof M Hix Zoology Dept University of Nairobi for their help obtaining the animals. This work was supported by grants from the Swedish Natural Science Research Council the Magn Bergvall Foundation

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ıtimation of cerebral extraction of circulating ımpounds by the brain uptake index method fluence of circulation time volume injection ıd cerebral blood flow

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HARDEBO J E. & NILSSON B Estimation of cerebral extraction of circulating compounds by the brain uptake index method: Influence of circulation time volume injection and cerebral blood flow Acta Physiol Scand 1979 107 153–159 Received 3 April 1979 ISSN 0001-6772 Department of Histology and Neurology and Laboratory of Experimental Brain Research University of Lund, Sweden.

The Oldendorf "brain uptake index" method for estimating and characterizing the transport of circulating substances across the blood-brain barrier in the rat has been widely applied The present study illustrates that minimizing the volume injected into the circulation to 10 µl or less is necessary Otherwise the flow artefacts, induced by the injected volume and the rate of injection, will influence the index. This principle is also relevant to techniques using intracarotid injections for the direct study of cerebral blood flow The study also elucidates the need for reducing the circulation time after injection to about 5 sec, to avoid artefacts due to metabolism and back diffusion to the circulation of the substances studied.

Key words. blood-brain barrier cerebral blood flow cerebral extraction

method originally described by Oldendorf has een idely applied for estimating the transport cross the blood-brain barrier (BBB) of various substances (Oldendorf 1971 1973 Wade & Katzman 975 see also Oldendorf & Szabo 1976 Pardridge Oldendorf 1977) The method in olves injection nto the common carotid artery in the rat of bolus f the radiolabelled test substance together with ighly diffusible reference substance usually water The animal is decapitated after 15 and the ercentage of residual test substance in the ipsilat ral brain hemisphere relativ to the eference substance is expressed as the brain uptake index BUI)

Critical analyses of this technique have shown that BUI is not strictly quantitativ measure of brain uptake function, mainly since the uptake of the reference substance is omplex function being influenced by both diffusion limitation and back diffusion (clearance) of the substance and thus highly dependent upon cerebral blood flow (CBF) (Bolwig & Lassen 1975 Bradbury et al. 1975 Oldendorf & Braun 1976 Raichle et al 1976)

The present study is an attempt to evaluate the importance of some reasonable modifications of the technique i e (1) reducing the volume of the bolus and thus the CBF response, (2) using a more diffusible reference substance than water and (3) reducing the time interval between bolus injection and tissue fixation.

(1) Injection of a large bolus volume may change the cerebral hemodynamics (Hardebo & Nilsson 1979). Since the uptake of the test and reference substances may be influenced both by the actual cerebral blood flow (CBF) and the available capillary exchange surface the effects on CBF and intravascular pressure of various volumes of the injected bolus were studied and related to the BUI

(2) Ideally blood-brain equilibration of the reference substance should occur instantaneously and the substance be virtually totally extracted from the brain circulation In the present study ethanol was chosen as reference substance since it is more freely diffusible across the BBB than water (Raichle et al. 1976)

(3) In the original experiments (Oldendorf 1970

1971) the animal was decapitated 15 s after injection This interval was an estimation of the time required for wash out of the test substance from the blood compartment after a single capillary passage However as shown by Furlow & Bass (1976) a non-diffusible substance appears on the venous side (external jugular vein) already after a few seconds with a peak after 2 5 s It is almost totally washed out after 5–6 s and reappears due to recirculation after 7–8 s Hence a time interval of about 5 s between injection and decapitation should be preferable At any prolongation of the interval the BUI obtained will not only reflect brain uptake but also clearance from the brain into the blood stream and may as well be influenced by brain metabolism of the test substance

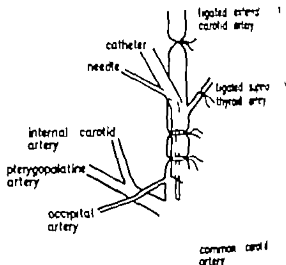


Fig 1 Position of catheter for infusions in the external carotid artery The infusions are carried into the internal carotid artery and in addition to the (external) pterygo-palatine and occipital arteries. Position of needle for measurement of intracarotid pressure at the level of the carotid bifurcation is indicated

METHODS

Preparation of animals The experiments were performed on male rats weighing 300–350 g Wistar rats were used for determination of cerebral blood flow and Sprague-Dawley rats for BUI studies. The rats had free access to pellet food and tap water until operation Anesthesia was induced with ~3% halothane to allow tracheotomy and immobilization with tubocurarine chloride (1 mg/kg i v) Halothane 0 6–0 8% was given during the operation procedure then withdrawn The animals were artificially ventilated on 70% N_2O and 30% O_2 (Braun) the $PaCO_2$ being adjusted to about 35 mmHg The body temperature was kept at 37°C by means of intermittent heating

Cerebral blood flow In 4 animals catheters were placed just distal to the aortic bifurcation via the femoral artery on one side for recording of the systemic blood pressure (AoP) via a pressure transducer (Elema Sweden) and sampling of arterial blood for blood gas measurements (BMS Mk II Radiometer Copenhagen) One femoral vein was cannulated for infusion of fresh donor blood during the period of blood sampling Both external carotid arteries were cannulated as described below The intracranial pressure (ICP) was measured through a needle inserted into the cisterna magna connected to a pressure transducer (Elema)

CBF was continuously measured with the technique developed by Nilsson & Siesjö (1976 1979) Thus the cerebral venous outflow was measured by drop counting in a closed circuit diverting blood from one retroglenoid vein during compression of the contralateral vein and returning the blood—drained from the forebrain on both sides—through a catheter in the external jugular vein on one side into the central venous system The outflow volume was calculated by the use of a diagram relating drop rate to volume It was controlled that hemodilution up to 40% (volume) with Krebs-ringer buffer solution did not increase the drop volume more than 5% Hence no attempts were made to correct for the possible influence upon drop volume of the infused solution

The outflow volume was converted into CBF values expressed in ml/100 g × min by measurement of the cerebral arterio-venous difference in oxygen content $(AVDO_2)$ in the control situation Assuming a cerebral oxygen consumption (CMRO) of 7.58 ml/100 g × min (Nilsson & Siesjö 1976) the CBF (in ml/100 g × min) could be calculated from 7 58/AVDO and related to the actual venous outflow volume

The effect on the CBF as reflected in the cerebral venous outflow rate as well as the effect on ICP were recorded following simultaneous infusion into each external carotid artery of 10 25 or 40 µl during 1 s of Krebs-Ringer buffer solution or blood Several experiments were run in each animal but a new experiment was not undertaken until the venous outflow rate had returned to control level Adequate reactivity of the CBF to hypercapnia was found as tested in each animal after the experiments had been completed

Intracarotid pressure In one group of 4 animals a needle was inserted into the external carotid catheter on one side with the opening just distal to the carotid bifurcation (Fig 1) The outer diameter of the needle was 0 1 mm compared to the inner diameter of the catheter 0 5 mm this allows a free flow past the needle during the infusion In addition a catheter was placed in to one femoral artery for recording of AoP and sampling of arterial blood for blood gas measurements These animals were used to measure the effect on intracarotid pressure (CarP) during the various intracarotid infusions (10 25 or 40 µl during 1 s)

Brain uptake index After ligation of all branches of the main trunk of the right external carotid artery it was cannulated centripetally in 71 animals with the catheter opening placed within one mm distal to the carotid bifurcation

...le 1 Changes in CBF (cerebral blood flow as determined by the cerebral venous outflow rate), ICP ...racarotid pressure), CarP (intracarotid pressure at the level of the carotid bifurcation) and AoP ...stemic blood pressure measured at the level of the aortic bifurcation) induced by an infusion of 10, 25 or ... µl during 1 s of Krebs Ringer buffer solution or blood into each internal carotid artery

...an ± S.E., number of observations within parentheses

...ered intracarotid ...sion during ...of	Duration of increase in CBF ( )	CBF maximum increase in % of the resting CBF	ICP maximum increase in % of the resting ICP	Increase in CarP (mmHg)	Increase in AoP (mmHg)
µl					
Buffered solute	5 8±0 4 (10)	6 7±0 7	3 1±0 8	4.0±0 3 (23)	1.2±0.4
Blood	5 7+0 5 (7)	3 3±0..	3.0±0.7		1.3±0 5
µl					
Buffered solute	10 6±1 2 (7)	15.9±2	12 4±0.3	12.2_1.2 (23)	4.6±1.0
Blood	10 8 0 7 (5)	9 3±0 9	11.6±0 7		4 8_1 1
µl					
Buffered solute	18 9 1 7 (8)	28 0±1.8	26.4±2 5	25 4±2.5 (16)	7.2±0 4
Blood	17 5 1 1 (6)	17 7±1 1	26 ±3 1		8 0±1.0

...ections through the catheter in the external carotid ...re then carried into the internal carotid artery supplying ... forebrain (and in addition to the territory of the extra... pterygopalatine and occipital arteries) (Fig. 1). A ...theter was also placed into one femoral artery for sampling of arterial blood for blood gas measurements. Determination of BUI ... performed by intracarotid infusion during 1 s of 10 or 40 µl of Krebs-Ringer buffer solution containing ... mixture of the reference substance ^{14}C ...thanol (Amersham, specific activity 27 1 mCi/mmol, concentration 100 nmol/ml, and either of the 3 different ...st substances L-noradrenaline-7 ^{3}H (NA) (New England Nuclear, specific activity 9 1 Ci/mmol, concentration 10 nmol/ml), ^{3}H-inulin (Amersham, specific activity ... 9 Ci/mmol, concentration 120 nmol/ml) or ^{3}H ...ater (Amersham, 5 Ci/ml, concentration 7 5 nmol/ml). ...rculatory arrest was induced by an intravenous injection of saturated potassium chloride 5 or 15 ... after administration of the bolus, the animal was instantaneously decapitated and the head frozen in liquid nitrogen. Various re... ...ions supplied by the internal carotid artery on the injected side (frontal, parietal, frontal part of occipital ...rtex, thalamus and caudate nuclei) were chiseled out ... cryostat at −15°C, weighed and placed in liquid scintillation vial containing 1.0 ml Soluene (Packard). When the tissue had been dissolved, 10 ml of Instagel was added. The tissue samples from each animal were counted for β-emissions in ... Nuclear Chicago liquid scintillation counter. Quench correction was performed along conventional principles. According to Oldendorf (1971), the brain uptake index (BUI) was calculated from the specific activity of ^{3}H (test substance) and ^{14}C (reference substance) in the tissue and in the injected mixture as follows

$$BUI = \frac{\text{tissue } {}^{3}H/\text{tissue } {}^{14}C}{\text{mixt. } {}^{3}H/\text{mixt. } {}^{14}C} \times 100$$

Statistics. Differences between mean values were assessed by the Student' t-test for unpaired data.

RESULTS

Cerebral blood flow and intracranial pressure. The resting mean (± S.E.) AoP was 120±5 mmHg, resting ICP 12.8±1.3 mmHg, CBF 73 7±2.2 ml/100 g × min, $AVDO_2$ 10.3 ±0.31 ml/100 ml, PaO_2 104±3 mmHg, $PaCO_2$ 35.3±1.2 mmHg and pH 7.34±0.16. The effects of intracarotid infusions are listed in Table 1 and illustrated in Fig. 2, showing data of representative experiments with an infused volume of 10 and 40 µl, respectively, of buffered solute. Infusion of 10 µl buffered solute during 1 s induced a very slight increase in CarP and CBF and a small change in ICP. AoP was almost unchanged. Increasing the infused volume over 25 to 40 µl gave rise to more pronounced and prolonged increase in CBF, ICP and CarP. The increase in intraarterial pressure caused by the infusion was even reflected within the vascular tree at the level of AoP measurement. The CBF had not returned to a resting level until about 20 s after injection of 40 µl of buffered solute. When blood substituted solute as infusate the CBF changes were less pronounced, evidently due to absence of hemodilution, while the effects upon CarP, AoP and also ICP were similar to those induced with buffered solute (Table 1). These findings thus show the purely pressure-induced changes in CBF and ICP. A transient fall in CBF and ICP was observed upon infusion of 25 or 40 µl buffered solute or blood a few seconds after the initial increase (Fig. 2). This may be due to an autoregulatory vasoconstriction.

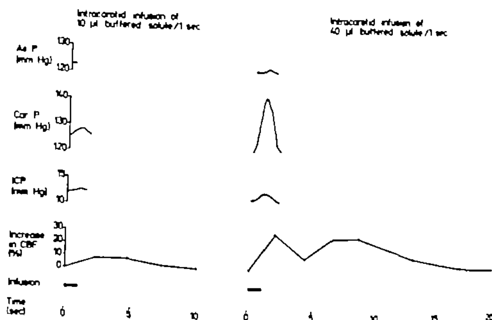


Fig 2 Representative experiment performed in one animal (intracarotid pressure was measured in a separate animal) illustrating changes in cerebral blood flow (CBF as reflected by the cerebral venous outflow drop rate), intracranial pressure (ICP measured in the cisterna magna) intracarotid pressure (CarP measured at the level of the carotid bifurcation) and aortic (systemic) blood pressure (AoP) induced by an intracarotid infusion on each side of 10 or 40 µl Krebs-Ringer buffer solution over 1 s

Brain uptake index The BUI in a brain hemisphere for inulin NA and water after various circulation times (5 and 15 s) and injected volumes (10 and 40 µl) are listed in Table 2 Injection of 40 µl gave a significantly higher index than 10 µl for water A similar tendency was noticed for NA and also inulin Further 15 s circulation time gave significantly lower index values than 5 s for NA

DISCUSSION

According to the original technique (Oldendorf 1970 1971) the radiolabelled mixture in an aqueous solution is injected through a needle [illegible] the common carotid artery on one side A high rate of injection is chosen to minimize mixing with circulating blood during the brain passage thereby preventing from binding of the test substance to plasma proteins Of the bolus mixture of 200 µl injected within approx 0 25 s about 40 µl is estimated to enter the internal carotid distribution (Oldendorf 1971) However the blood flow to one hemisphere is normally only about 10 µl/s (equivalent to [illegible] ml/100 mg and min) (Nilsson & Siesjö 1976) for a 0 75 g brain hemisphere Therefore it is not surprising to find that 40 µl of an aqueous solution [illegible]

Table 2 *Brain uptake index (BUI) for tritiated water l noradrenaline and inulin using* [14]*C-ethanol as reference substance after intracarotid injection of a radioactive bolus of 10 or 40 µl and circulation time of 5 or 15 s*

The values represent mean uptake (±S E) in regions supplied by the internal carotid artery from 6 animals (for [illegible] µl/15 s 5 animals) Comparison of mean values according to Student's *t* test for water 10/40 µl *0 01<P<0 05 for l-noradrenaline 5/15 s P<0 001 otherwise non-significant difference

	10 µl		40 µl	
Substance	5	15	5 s	15
Water	8[illegible]8±1[illegible]	79 4± [illegible] 1	87 9±1.5	85 4±1 1
l Noradrenaline	5 41±0 [illegible]	3 36±0 31	5 84±0 15	3 54±0 20*
Inulin	1 64±0 05	1 64±0 10	1 70 0 04	1 68±0 05

ted over 1 down to the external carotid bilater y to reach the internal carotid distribution in- ces a substantial increase in CBF and ICP hough part of the infused volume enters the ex cerebral circulation (via the pterygopalatine and cipital arteries) It is notable that the flow in- case persists beyond the point of tissue sampling ed in Oldendorf's model i.e 15 s after injection g 2) 50 μl injected over approximately 0.5 s rectly into one carotid internal artery in its flow rection (the common and external carotid ligated) ases an even more marked increase in CBF and rebrovascular volume (Furlow & Bass 1976) as ll probably the volume injected in the original ldendorf technique and in e.g. the CBF technique sed by Crone (1963) and Hertz et al (1977) Apart om hemodilution this increase in CBF is caused by pressure-induced distension of vessels which ay open closed microvessels thereby increasing e available capillary exchange surface It should e noted however that, for technical reasons, the resent CBF studies had t be undertaken with ilateral injection, thus not exactly mimicking the ffects of unilateral injections used for BUI studies

E idently the use of water as reference substance ll give BUI alues that are overestimated as compared to the alues obtained with a more diffusible eference substance The higher BUI values for water found in the present series when a bolus of 40 μl was used (Table) should primarily be explained by the increase in CBF provoked by the injection This explanation would however imply an increasing flow dependence of the extraction also for the more diffusible substance (used as reference) which is not supported by the findings by Raichle et al (1976). These authors found that about 97% of an injected bolus of labelled water was e changed with brain at a CBF of 50 ml/100 g min, while the comparable figure for ethanol was 98%. However the extraction as reduced to about 77 and 87% respectively at CBF of about 100 ml/100 g min hich should lower the ethanol/water uptake quotient. Possibly other factors come into play Thus, an increased back diffusion of ethanol during the clerance phase may influence the result even during the 5 interval The finding of an unchanged BUI for inulin at 15 compared to 5 further indicates that back diffusion of the reference substance is involved Thus definite washout of inulin should ha occurred during the 15 s period and yet the quotient inulin ethanol is unaltered With increasing flow represented here by 40 μl instead of 10 μl the uptake will be overestimated even when ethanol is used. Using tritiated water as standard, Oldendorf (1971) found a mean BUI in the hemisphere of 4.5% for NA at a circulation time of 15 s, whereas in the present experiments, using ¹⁴C-ethanol as standard (10 and 40 μl volume respectively 15 s circulation time) values of 3.36–3.54 were obtained Oldendorf's (1971) and the present values for inulin were 1 99 and 1 64–1 68, respectively

The significantly lower BUI value for NA after 15 circulation time compared to the value after 5 s elucidates another mechanism that necessitates reduction of the circulation time It is generally considered that circulating neurotransmitter monoamines are not able to reach the brain parenchyma (for review see Edvinsson & MacKenzie 1976, see also Hardebo et al. 1979a) To a high extent, the structural unity of the endothelial lining at the level of the BBB (Reese & Karnovsky 1967) prevents the polar and water-soluble NA from being extracted from the brain circulation as reflected in the low BUI after 5 s circulation time The few per cent of NA that do penetrate into the endothelial cells of brain microvessels and—at the level of brain parenchymal arterioles and pial vessels—further into the smooth muscle cell layer are metabolized by monoamine oxidase and catechol-O-methyl transferase present in these structures (Bertler et al 1966, Spector et al 1977 Hardebo et al. 1977 1979b) probably constituting a further enzymatic barrier for monoamines That a small fraction of NA actually can reach the brain vascular smooth muscle cells and induce a transient vasoconstriction (before the amine is metabolized)—if administered in sufficient amounts —is consistent with the finding of a shortlasting reduction n CBF upon intracarotid injection in the rat of a bolus of NA (Hardebo Nilsson & Owman 1979) It is highly likely that the main explanation for the lower BUI value for NA at 15 s—apart from simple back-diffusion to the circulation and washout from the brain—is metabolism in the brain vessel wall and subsequent clearance back to the circulation Rapid degradation by catechol-O-methyl transferase may be mainly responsible since metabolism via monoamine oxidase is a slower process. It should be noted that reduction of circulation time does not substantially influence the BUI for water i.e a non-metabolized substance However as a general principle, it

should be advantageous to avoid the possible influence of secondary metabolic effects by using a short circulation time

In the present series of experiments a further modification of the Oldendorf technique comprised injection into the internal carotid artery via the cannulated external carotid and not through a needle in the common carotid artery This procedure eliminates any interruption or diminution of the blood flow through the internal carotid artery and also lowers the risk of vasospasm of this artery frequently occurring when the common carotid artery is manipulated

CONCLUSIONS

The present study clearly illustrates that minimizing the volume injected into the rat brain circulation is necessary Otherwise the flow artefacts induced by the injected volume and the rate of injection will influence the BUI This principle should also be relevant to techniques using intracarotid injections for the direct study of CBF 10 μl was the smallest precise volume that could be administered in our model if possible in the rat the use of even smaller volumes would be an advantage The results further elucidate the need for reducing the circulation time from 15 s to about 5 s (cf Oldendorf & Braun 1976) to avoid as far as possible effects of clearance and metabolism The use of ethanol as reference substance is advantageous even if it is maybe not the ideal substance

This work was supported by grants from the Swedish Medical Research Council (grant No 04X 732 and 14X 263)

The skilful technical assistance of Mrs Camilla Hultman is gratefully acknowledged

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;omparative study on the uptake and subsequent carboxylation of monoamine precursors cerebral microvessels

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HARDEBO J E, FALCK, B & OWMAN CH A comparative study on the uptake and subsequent decarboxylation of monoamine precursors in cerebral microvessels. Acta Physiol Scand 1979 107 161 167 Received 3 April 1979 ISSN 0001-6772. Departments of Histology and Neurology University of Lund, Sweden.

The endothelial cells and pericytes of brain microvessels (capillaries and small veins) are equipped with an enzymatic barrier impeding the passage of circulating amino acids, such as amine precursors into the brain. The properties of this mechanism was studied in brain slices and isolated microvessels from various species including man and also fetal material following incubation in dihydroxyphenylalanine (DOPA), 5-hydroxytryptophan (5-HTP) and dihydroxyphenylserine (DOPS). A stereospecific, energy-dependent uptake leading to accumulation in the brain microvessel walls was found in all species studied this proces was found to exist already prenatally The capacity of decarboxylation the second step in the trapping mechanism at the blood-brain interphase showed considerable species variation The enzyme was present also in fetal brain microvessels. Inhibition experiments provided support for the presence of monoamine oxidase but absence of catechol-O-methyl transferase, in the microvessel walls.

Key word brain microvessels, aromatic aminoacid decarbo ylase monoamine xidase, blood-brain barrier

he microvessel (capillaries and small veins) in the ntral nervous y tem are capable of taking up and carboxylat monoamine precursors, such as L 3 dihydroxyphenylalanine (L-DOPA) (Bertler et al 64 1966 Owman & Rosengren 1967 Bartholini al 1971 Dowson & Laszlo 1971 Wade & Katz an 1975a, b) L 5-hydroxytryptophan (L 5-HTP) lertler et al 1964) and 3 4-dihydroxyphenylserine DOPS) (Constantinidis et al. 1975) Thi presence f aromatic L-aminoacid decarboxylase (AAD) e endothelial cells and pericytes of these essels onstitute an enzymatic barrier imped ng the pasage of the monoamine precursors into the brain, as hown in the rat and mouse (Bertler et al 1966) The present study wa ndertaken to investigate he presence of the uptake mechanism for monoamine precursors, as well a the AAD activi-) in brain microvessels of arious species includ- ng man The appearance of these functions in the fetal brain was also studied.

MATERIALS AND METHODS

Animals The majority of the study was performed on adult animals of either sex: 8 albino mice 67 Sprague Dawley rats, 8 hamsters 6 guinea-pigs, 6 albino rabbits, 4 cats, 4 dogs, 4 pigs 4 cows, 3 baboons and brai tissue from 5 humans (obtained during neurosurgical operations) The laboratory animals had free access to standard pellet food and water They were killed under light ether or nembutal anesthesia. In addition, fetuses of various age from rat (32 fetuses from 4 animals), rabbit (8 from 3 animals) and human (hich pregnancy wa interrupted at 20 and 22 eeks of gestation) was studied Blood vessels appear in the central nervous ystem of the rat on day 1 14 of gestation (Bär & Wolff 1972) In the rabbit at 11 days of gestation (Donahue 1964), and are present in human fetuses at the 10th eek of gestation (Povlishok et al. 1977)

Incubation of ti sue lic One mm thin slices of the parietal corte caudate nucleus cerebellum spinal cord and hea from 4 animal of each species studied (from temporal corte frontal corte or cerebellum of man) ere cut ith razor blade and transferred to incubation ials containing ice-cold Krebs-Ringer buffer solution Slices ere also taken from the brain hemispheres—pref

erentially cortical tissue—of fetuses of various ages from rat rabbit and man The buffer solution had the following composition (mM): NaCl 118 KCl 4.5 $CaCl \times H_2O$ 2.5 $MgSO \times 7H_2O$ 1 0 $NaHCO_3$ 25 KH PO 1 0 to which was added 1 mg/ml glucose and 0.2 mg/ml ascorbic acid The solution was continuously aerated with a mixture of 95% O_2 and 5% CO_2 giving a pH of 7 4

The vials were placed in an incubation bath at 37°C for preincubation during ?0 min in the presence of the decarboxylase inhibitors, carbidopa or benserazide (10⁻ M) followed by incubation during ?0 min after the addition of the various monoamine precursors at a dose of 1 10 or 100 μg/ml L DOPA D-DOPA (+)-erythro-DOPS (−)-erythro-DOPS (±)-threo-DOPS and L 5-HTP In some of the incubations the monoamine oxidase inhibitor nialamide (10^{-4} M) or the catechol-0-methyltransferase inhibitor U-05 1 (10⁻ M), replaced carbidopa or benserazide Control slices were incubated without addition of any drugs.

The uptake of L DOPA L 5-HTP and (+)-erythro-DOPS (in the presence of carbidopa) was further characterized in the presence of ouabain (10^{-4} M) at low sodium and high potassium concentrations (in the buffer solution KCl substituted for all NaCl) under combined anoxia (continuous bubbling with 95% N and 5% CO_2) and absence of glucose In the presence of dinitrophenol and also at 0°C

Fluorescence microscopy Following incubation the various tissue pieces were frozen to the temperature of liquid nitrogen and further processed for fluorescent monoamine histochemistry according to the Falck Hillarp method (Björklund Falck & Owman 197?) The paraformaldehyde used in the histochemical procedure had previously been equilibrated with air of 70% humidity The formaldehyde-induced histochemically vi ible fluorophores of noradrenaline adrenaline, dopamine DOPA and DOPS are indistingui hable since they have the same spectral characteristics and exhibit a green light under the optical conditions used (Björklund et al 197?) 5 HTP and 5-hydroxytryptamine exhibit a yellow rapidly fading light under these conditions (Björklund et al 197) When evaluating the changes in fluorescence intensity after incubations under various conditions the material to be compared was always freeze-dried and further processed on the same occasion

Is lation f erebral micros el Whole brains from rats were used After removal they were kept in an ice cold 67 mM phosphate buffer (pH 7 4) throughout the procedure The meninges including the pial membrane and its vessels were carefully torn off and the choroid ple uses were removed White matter including the whole brain stem was d ssected away Ti sue obtained from 4–6 rats was chopped with a razor blade and then gently further disrupted by some 10 strokes up and down through 3 different 10–?0 ml plastic syringes eq ipped with a nylon net (pore size 1 000 μm 500 μm and ?0 μm respectively) glued to their open cut end The material was subsequently homogenized by hand with a loosely fitting Teflon pestle in a smooth gla tube (0 1 mm clearance) The homogenate was washed through one nylon sieve with ...5 μm pore size The material remaining on the sieve after e tensi e washing wa re-homogenized and re-sieved 3–4 times through the same carefully washed sieve The tissue passing these sieves was collected and sieved through another sieve with ?? μm size The material remaining on this sieve after ... was re-homogenized and re sieved in the same ... washed sieve The tissue fraction remaining on the ... sieve after extensive wa hing consists of ... venules and a few larger vessels, whereas most of larger vessels and vessels branching off ... small vessels, as well as a few small clusters of ... and glia remain on the ...5 μm sieve (Hardebo et al ...) Neurons, glia small microvessel segments, and ... lar fragments pass the 75 μm sieve The capillary fraction is pure with regard to vessels and is only contam... with a few glial endfeet stuck onto the vessel wall ... debo et al 1977) and possibly perivascular nerves ... yield of microve sels is less than 1/1 000 of the original weight of the grey matter Brendel, Meezan & Ca... (1974) have shown that a mixed fraction of intracerebral vessels prepared under similar conditions is metabolical ly active

Incubation of isolated microvessels The microvessel fractions were transferred to incubation vials ... ice-cold Krebs-Ringer buffer solution (composition ... above) The vials were placed in an incubation bath ... allowed to equilibrate for ?0 min at 37°C in the presence of the decarboxylase inhibitor benserazide (10^{-4} M) before the incubation which was started by adding ³H-L-DOPA ... the solution The tissue preparations were incubated ... 15 min at a temperature of 37°C The buffer solution ... continuously aerated with 95% O_2 and 5% CO_2 ... Incubation was terminated by transferring the tissue ... ice-cold buffer solution The microvessel fraction was collected by centrifugation at 4°C at 110 g and ...ashed ... in isotope-free cold buffer for each 10 min

In order to further characterize the uptake ... microvessel fractions were also incubated in the presence of ouabain (10⁻ M) and at 10°C

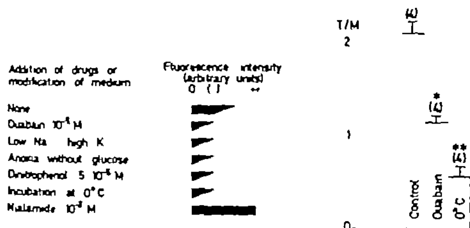
Measurement of rad activity After incubation ... microvessel samples were weighed and transferred ... liquid scintillation vial They were solubilized in ... Soluene (Packard) and liquid scintillation counting ... performed in 10 ml Instagel (Packard), ... Appropriate samples (...5 μl) of the incubation medium ... corrections were obtained according to ... samples

Drugs L DOPA and D-DOPA (Sigma), (±)-threo-DOPS (+)-L and ( )-D-erythro-DOPS (all gifts from ...), carbidopa (MSD), benserazide (Roche) L 5-HTP (Sigma) carbidopa (MSD), ... (Roche) ouabain (Sigma) nialamide (Pfizer), and ... H L DOPA (Radiochemical Centre Amersham, ... mmol)

Statistics Mean ... lues were compared according to Student s t-test for unpaired data

RESULTS

Fluorescence microscopy f the variou brain regions from control slices incubated in buffer solution alone howed a dense netw rk of delicate catecholamine-containing nerve terminals ...



I Accumulation of L-DOPA in the walls of microvessels in rat parietal cortex slices pretreated with benserazide M and incubated in the presence of 10 μg/ml L DOPA alone and after addition of drugs or modification of the ium (following decarboxylase inhibition by 10^{-4} M benserazide). For comparison the fluorescence intensity owing pretreatment with maleimide instead of benserazide is shown (the accumulated fluorophore now consists of . *II* Tissue/medium ratio (*t/M* mg tissue per μl medium) following 15 min incubation of isolated cerebral ovessels with ³H-L-DOPA (and after decarboxylase inhibition by 10^{-4} M benserazide) under control conditions °C) and in the presence of ouabain (10^{-4} M) or during hypothermia (0°C). Values are means ± S.E. number of ments in parenthesis. Control vs experimental according to Student' *t*-test. *0.01<*P*<0.05 001<*P*<0.01

reen fluorescence in the cerebellum only a small mber of isolated green-fluorescent axons were served. The parenchymal blood vessels were ipped with varying amounts of sympathetic rves. In the heart tissue adrenergic nerves were nt in the myocard and in association with the ood vessels. All tissues showed a slight diffuse, n-specific greenish background fluorescence. e vessel wall proper was in all region essentially non-fluorescent except for the intense autofluorescence in the internal elastic membrane of rger pial arteries.

After incubation with the various monoamine ecursors (in the presence of the decarboxylase hibitors, carbidopa or benserazide) an accumulation of fluorophore weak but above the diffuse ckground fluorescence was seen in the brain icrovessel walls (endothelial cells and pericytes of pillaries, and small veins) to a similar degree in l adult species studied and irrespective of whether nserazide or carbidopa was used as decarboxylase inhibitor. The intensity of the general ckground fluorescence was also increased. No earcut accumulation above the diffuse background fluorescence had occurred in the walls of the brain parenchymal arterioles pial vessels, or heart vessels. Of the various amino acids tested (at an identical concentration in the incubation bath) L-DOPA induced the strongest fluorescence in the microvessel wall of brains from adult and newborn animals. L 5-HTP and (+)-erythro-DOPS a somewhat weaker fluorescence whereas the fluorescence induced by D-DOPA, (−)-erythro-DOPS or (±)-threo-DOPS was considerably weaker. The discrepancy in fluorescence intensity between L-DOPA on one hand and L 5-HTP and (+)-erythro-DOPS on the other is well explainable by the varying fluorescence yield in the standard formaldehyde reaction used (Björklund et al. 1972). Also in brain slices from fetal and newborn animals a clear-cut uptake of the monoamine precursors was seen.

Fig. I and *b* show the inhibitory influence on the uptake of *L-DOPA* (in the presence of a decarboxylase inhibitor) into brain microvessel walls by various drugs or by modification of the medium as studied in brain slices as well as in isolated fractions of microvessel (using radioactive L DOPA). The pattern of inhibition, being the same for L 5-HTP and ( )-erythro-DOPS indicates the presence of an

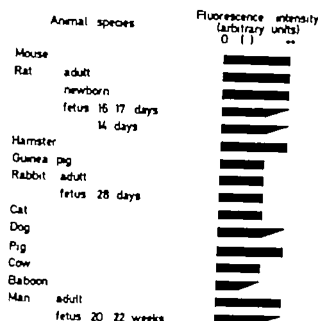


Fig 2 Accumulation of fluorophore (L DOPA and dopamine) in the microvessel wall of brain cortical slices from various species (adult unless otherwise stated) incubated for 30 min in the presence of 10 μg/ml L DOPA following monoamine oxidase inhibition (10^{-4} M nialamide) and processed according to the formaldehyde histofluorescence method The comparison refers to the combined presence of L DOPA and dopamine (formed by decarboxylation within the microvessel wall) since the fluorophores are undistinguishable under the optical conditions used Tapering part of the bars indicate intermediary intensities

energy-dependent uptake mechanism into the cells of the brain microvessel wall

Inhibition of monoamine oxidase by nialamide (but with intact decarboxylase activity) clearly enhanced the accumulation of the fluorophores in the microvessel wall as evidenced by a stronger fluorescence intensity in relation to the background fluorescence as compared to that obtained after inhibition of the AAD activity (Fig. 1*a*) This provides indirect evidence for the presence of microvascular AAD activity in all species studied as well as the presence of a subsequent breakdown mechanism via monoamine oxidase in these vessel walls However considerable species differences were now revealed indicating variations in AAD activity (Fig. 2) Also in slices from fetal brains inhibition by nialamide revealed an enhanced accumulation in the microvessel wall indicating the presence of microvascular AAD activity already at this age (Fig. 2) On the other hand inhibition of catechol-O-methyl transferase by U-0521 did not affect the accumulation of fluorophore in the microvessel wall

DISCUSSION

The integrity of the endothelial lining along with paucity in transendothelial vesicular transport and absence of fenestrae in brain vessels (Reese & Karnovsky 1967 Westergaard & Brightman 1973) constituting the morphological blood-brain barrier impedes the passage of a large variety of compounds from the circulation into the brain However substances may pass this barrier by virtue of lipid solubility or with the aid of carrier mechanisms The present study provides evidence for the existence in various species including man of an energy-dependent uptake mechanism for the immediate precursors to neurotransmitter monoamines at the level of the endothelial cells and pericytes of brain microvessels in contrast to the endothelium of brain parenchymal arterioles The study does not show whether the uptake occurs across the luminal or the abluminal side of the microvessel wall or both An avid uptake of L-DOPA in vivo into brain microvessel walls of the rat has been reported following intracarotid administration of radioactive L DOPA (Oldendorf 1971 Wade & Katzman 1975*a* own unpublished observations) but simple diffusion also contributes to some extent (Wade & Katzman 1975*a*) An energy-dependent uptake of L DOPA into rat brain slices (thus reflecting both an uptake into neurons and into vessel walls) has also been characterized (Yoshida et al 1963*a* and *b*) in a similar way as presently for microvessels Also in the newborn and fetal brain is this noticed

The present uptake mechanism at the level of the blood-brain barrier is supposed to be shared with other neutral amino acids as shown for the phenylalanine methionine histidine cysteine valine isoleucine leucine tryptophan and threonine (Chirigos et al 1969 Oldendorf 1973 Pardridge 1977) Moreover this movement across the membranes is bidirectional (Christensen 1969 Wade & Katzman 1975*b*) which is consistent with the finding that L DOPA is readily taken up into the microvessel wall also when administered into the brain parenchyma (Bertler et al 1966) A facilitated diffusion of L DOPA into the microvascular endothelial cell and pericytes would mean that the uptake is

— along a concentration gradient so that the y from the circulation—or brain parenchyma rtler et al 1977)—ceases when the intracellular centration of free L DOPA is equal to that on outside of the cell membrane That his-emically visible accumulation of fluorophore e that of the background fluorescence in fact is med, even when the conversion to dopamine efficiently been prevented by a decarboxylase bitor indicates that some kind of intracellular ing of L-DOPA occurs in the microvessel wall other possibility is that the ptake is not solely ple facilitated diffusion, but has a component of active transport of the uphill" type as indicated the mode of inhibition found in the present in-bation studies, in line with the reasoning made by de & Katzman (1975)

Of the two decarboxylase inhibitors used espe lly carbidopa has a tructural similarity with L DPA. It would, therefore be possible that this ibitor hares an uptake mechanism in common th L-DOPA acros the endothelial membrane nding to reduce the absolute amount of L-DOPA ing able to reach the endothelial cytoplasm when ese two agent are present together in the incuba-on bath Even so the uptake of L DOPA s still gh enough for adequate fluorescence histochemi-al visualization.

The degree of intracellular L-DOPA accumula-on as reflected in the ratio of radioactivity in the microvessel fraction and the medium, was less than hat reported for the uptake of noradrenaline into sympathetic nerves under similar conditions (Ed vinsson & Owman 1977 Alm et al 1979) but con-siderably higher than the extraneuronal amine up-take into smooth musculature (Alm et al 1979) The energy-dependency of the m crovessel uptake wa confirmed by the inhibition accomplished with hypothermia combined anoxia and glucose depri-vation, or uncoupling of the oxidative phosphoryla-tion ith dinitrophenol The result from the in-cubation with low sodium and high potassium, as ell as in the presence of ouabain, uggest that the uptake is linked with N /K -dependent ATPase The uptak of monoamine precursors i stereo-selective since L DOPA and ( )-erythro-DOPS ere taken up more readily than D-DOPA, (−)-erythro-DOPS and ( )-threo-DOPS in brains from adult and newborn animals (see also Bertler et al. 1966, Oldendorf 1973 Constantinidis et al 1975) However the possible existence of a stereospe cificity was not possible to establish in the fetal material

Pretreatment with the monoamine oxidase in hibitor nialamide enhanced the accumulation in the endothelial cells and pericytes of the microves-sel wall. This finding offers support for the presence of monoamine oxidase in these cells (Bertler et al. 1966, Spector et al. 1977 Lai & Spector 1978 Har debo et al 1977 1979) When studying the effect of pretreatment with nialamide no decarboxylase in-hibitor was present in the bath. Hence the en-hanced accumulation of fluorophore in this situa-tion is primarily due to the formation of the corre sponding monoamine (dopamine 5-hydroxytryp-tamine or noradrenaline) from the respective pre-cursor (L-DOPA L 5-HTP or DOPS) within the microvessel wall It should be recalled that the fluorescence of the precursor and the correspond-ing monoamine are indistinguishable under the optical conditions used. The decarboxylase activity thus revealed was found to vary between species, as reflected in the difference in accumulation of fluorophore. This species difference agrees with findings from chemical determinations on isolated cerebral microvessels (Hardebo et al 1979) and in histofluorescence studies following administration of L DOPA in vivo (Langelier et al 1971) or after incubation of brain slices in the presence of the amino acid (Dowson & Laszlo 1971 Dowson 1973) Decarboxylase activity was indicated also in the microvessel wall of the newborn and fetal brain This supports the findings in vivo on rats (Kellogg et al 1973)

Inhibition of catechol-O-methyltransferase did not affect the accumulation of fluorophore in the microvessel wall, indicating that this enzyme is not present in the endothelial cells and pericytes (see also Hardebo et al 1979 cf Spector et al. 1977).

L DOPA, and probably 5-HTP too is found in the circulation (where the normal L-DOPA con-centration is about 3×10 M Hansson et al 1978) despite the presence of a considerable decar boxylase activity in many visceral organs. Apart from metabolizing circulating DOPA and 5-HTP that would otherw se have been taken up across the blood-brain barrier by the neutral amino acid carrier system, the decarboxylase activity in brain micro-vessel wall may be involved in a more complex enzymatic barrier mechanism for other circulating mino acids such as phenylalanine tyrosine and tryptophan or may be invol ed in a hitherto un

known metabolic pathway across the blood-brain interphase

The efficiency of the blood-brain barrier to impede the passage of L DOPA into the brain by decarboxylation In addition to the decarboxylation activity in the peripheral tissues have required high doses of L DOPA in the medical treatment of Parkinson s disease with disturbing side-effects as a frequent result Therefore a decarboxylase inhibitor (carbidopa or benserazide) effective in peripheral tissues as well as in brain microvessel walls but only to a negligible extent in the brain parenchyma is now added to L DOPA in the therapy Under these conditions the amine precursor can be administered in lower doses reducing the incidence of undesired side-effects

This work was supported by grants from the Swedish Medical Research Council (grants No 04X 732 and 04X 56)

The skillful technical assistance of Mrs Inga-Margit Hägg is gratefully acknowledged

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ıe vagal control of the felıne pylonc sphincter

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EDIN R AHLMAN H & KEWENTER, J The vagal control of the feline pyloric sphincter Acta Physiol Scand 1979 107 169-174 Received 5 April 1979 ISSN 0001-6772. Department of Surgery III University of Goteborg, Sweden

In acute xperiments on cats in chloralose anesthesia the effects of efferent and afferent electrical stimulation of the cervical vagi on an applied constant flow of saline through the feline pylorus was studied. The motor activity of the stomach was recorded simultaneously w th volume recording technique Efferent cervical vagal stimulation caused decrease in the transpyloric flow and an increased gastric motor activity In a few animals the decreased transsphincteric flow was preceded by short period of increased flow When the transpyloric flow was reduced by splanchnic nerve stimulation or noradrenaline infusion vagal nerve stimulation induced an increased flow through the pylorus indicating the presence of relaxatory fibres to the pylorus within the vagi. Electrical stimulation of the central end of the ipsilateral vagal nerve in the neck, with the contralateral vagal nerve left intact, resulted in decreased transpyloric flow and relaxation of the stomach. This response could be induced with or without intact splanchnic nerves, and disappeared when the intact contralateral vagus was cut It is concluded that the vagi mediate both xcitatory and inhibitory fibres to the pyloric sphincter in the cat. A vago-vagal excitatory reflex to the pylorus can be elicited by afferent vagal nerve stimulation together with vago-vagal relaxatory response of the stomach.

Key ord Pyloric sphincter gastric motility electrical stimulation, vagal nerves, cat

he presence of an anatomical well-defined pyloric phincter has been demonstrated in some species ut strongly questioned in others (Didio 1968 Edards 1968 and Kaye et al 1976) However forgersen (194) demonstrated the presence of uch phincter at the gastroduodenal junction of he cat Histochemical studies have verified a high umber of cholinergic ganglion cells and adrenergic erve terminals in this region compared with sur ounding part of the gastrointestinal tract indicat ing the presence of a specific sphincter region (Schardt et al 1974)

A high pressure zone at the gastroduodenal junc tion has been documented (Brink et al 1965). Antral peristalsis caused a decreased pressure within this zone while duodenal perfusion with amino acids glucose and fat increased the pressure (Fisher & Cohen 1973). Thus physiological characteristics for gastrointestinal phincter are fulfilled for the gastro-duodenal junction according to the definition given by Fisher & Cohen (1973)

In n tudies have indicated that the vagal nerves con ey nonchol nergic relaxatory fibres to the pylorus in dogs (Mir et al 1977) If these fibres are identical to those which mediate the noncholinergic non-adrenergic gastric relaxation is still unclear since volume recordings of gastric motility were not recorded simultaneously in their experi ments Whether the vagi convey nerve fibres to the pyloric region, which can control this sphincter independently of the gastric motor activity is not know n.

The aim of the present investigation was to study the effect of efferent and afferent vagal nerve stimulation on an externally applied flow of saline through the feline pyloric sphincter It was also judged to be of interest simultaneously to study the gastric motility in order to investigate whether the vagal nerves might exert specific effects on the pylorus separated from these on the stomach.

MATERIAL AND METHODS

The experiments ere performed in 77 cats of both sexes The animals ere fasted for about 24 h prior t the periment but had free access to water The anesthesia

Physiol Scand 1979 107 169–174

The vagal control of the feline pyloric sphincter

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In acute experiments on cats in chloralose anaesthesia the effects of efferent and afferent electrical stimulation of the cervical vagi on an applied constant flow of saline through the feline pylorus was studied. The motor activity of the stomach was recorded simultaneously with a volume recording technique. Efferent cervical vagal stimulation caused a decrease in the transpyloric flow and an increased gastric motor activity. In a few animals the decreased transsphincteric flow was preceded by a short period of increased flow. When the transpyloric flow was reduced by splanchnic nerve stimulation or noradrenaline infusion, vagal nerve stimulation induced an increased flow through the pylorus indicating the presence of relaxatory fibres to the pylorus within the vagi. Electrical stimulation of the central end of the ipsilateral vagal nerve in the neck, with the contralateral vagal nerve left intact resulted in a decreased transpyloric flow and relaxation of the stomach. This response could be induced with or without intact splanchnic nerves, and disappeared when the intact contralateral vagus was cut. It is concluded that the vagi mediate both excitatory and inhibitory fibres to the pyloric sphincter in the cat. A vago-vagal excitatory reflex to the pylorus can be elicited by afferent vagal nerve stimulation together with a vago-vagal relaxatory response of the stomach.

Key words: Pyloric sphincter, gastric motility, electrical stimulation, vagal nerves, cat

The presence of an anatomical well-defined pyloric sphincter has been demonstrated in some species but strongly questioned in others (Dedio 1968, Edwards 1968 and Kaye et al. 1976). However Torgersen (1942) demonstrated the presence of such a sphincter at the gastroduodenal junction of the cat. Histochemical studies have verified a high number of cholinergic ganglion cells and adrenergic nerve terminals in this region compared with surrounding parts of the gastrointestinal tract indicating the presence of a specific sphincter region (Schardt et al. 1974).

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In vivo studies have indicated that the vagal nerves convey noncholinergic relaxatory fibres to the pylorus in dogs (Mir et al. 1977). If these fibres are identical to those which mediate the non-cholinergic non-adrenergic gastric relaxation is still unclear since volume recordings of gastric motility were not recorded simultaneously in their experiments. Whether the vagi convey nerve fibres to the pyloric region which can control this sphincter independently of the gastric motor activity is not known.

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MATERIAL AND METHODS

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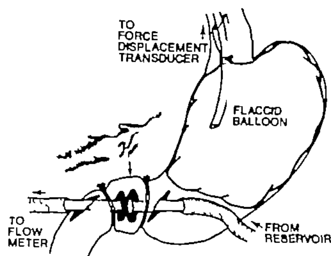


Fig 1 Schematic drawing of the technique used to study transpyloric flow Note histology sections from this region demonstrating a circular valve/sphincter

was initiated with ether and was continued with chloralose (50 mg/kg b w) slowly injected through one of the femoral veins The animals were placed on an operation table on a thermostatically regulated heating pad in order to maintain the body temperature at about +38°C A tracheal cannula was inserted to guarantee free airways A femoral artery was cannulated and connected to a pressure transducer (Statham p 23 DC) for continuous recording of the arterial blood pressure The abdomen was opened with a midline incision The catecholamine secretion from the adrenal medulla is known to be a potent intestinal inhibitor To eliminate this effect the arterial and venous vessels of the adrenal glands were ligated

Nerve operations and stimulation procedures Through the tracheostomy incision the cervical vagi were isolated from the sympathetic trunk and divided uni- or bi-laterally The cut ends of the vagi were inserted separately into insulated ring electrodes which were cautiously placed deep into the wound before nerve stimulation In 16 cats given efferent vagal nerve stimulation the splanchnic nerves were left intact in 5 animals and divided bilaterally in 11 animals at the preganglionic level subdiaphragmatically prior to the stimulation

In animals given unilateral afferent nerve stimulation the splanchnic nerves were left intact in 7 and divided bilaterally in 11 cats In 4 animals both splanchnic and vagal nerves were divided during the expt All nerve stimulations were performed using square wave pulses from a Grass stimulator (S4E) during 5 min The stimulation parameters (8–15 V 0.. 5 ms 8–12 Hz) were divided into 2 groups activating high (5 ms) and low threshold (0.. ms) efferent fibres selectively according to Martinson (1965*a*) The same groups of parameters were applied for the afferent nerve stimulations

Recording of transpyloric flow A non-compressible plastic tube (diameter 8 mm) was inserted through small longitudinal incision along the greater curvature on the anterior wall of the lower corpus region of the stomach and fixed with a loose ligature immediately proximal to the pylorus Consequently antral motor activity could not influence the transsphincteric flow A similar plastic tube was inserted through a longitudinal incision in the [illegible] portion of the duodenum opposite the sphincter The tube was brought up to the pylorus opposite the proximal tube In this position the distal tube was fixed by a firm ligature around the duodenum (see Fig 1)

A reservoir containing normal saline thermostatically regulated to be at body temperature was connected to the proximal tube The distal tube was connected to a photoelectric flowmeter which records volume changes of the transpyloric flow within 0–1 000 ml/min The saline from the flowmeter was collected and constantly perfused up to the perfusion reservoir Since the fluid surface of the reservoir was large (370 cm²), the inflow pressure during the sphincter could be kept almost constant by means of recirculation of the saline The pressure gradient between both reservoirs and the pylorus was variable The mean height between the reservoirs was 50 cm. To initiate the inflow a pressure of 70 cmH$_2$O was induced and immediately thereafter reduced to about 10 cmH$_2$O (Fig 2)

Recording of gastric tone A flaccid plastic balloon of about 1 000 ml volume was introduced orally into the corpus-fundus part of the stomach in the anesthetized animal The balloon was connected by a rubber tube to a water tank mounted on a weight recorder (Grass Force Displacement Transducer FT 10C) and connected to an amplifier The water level in the tank could be varied by changing the level of the weight transducer By opening the system with the tank at different water levels in relation to the stomach water was allowed to fill the gastric balloon In most expts such an hydrostatic pressure gradient was accomplished at 5 cmH$_2$O Recording of blood pressure intragastric tone and transpyloric flow were simultaneously recorded by means of a Grass polygraph (Grass S4E) (Fig 2)

RESULTS

Efferent vagal nerve stimulation Uni and bilateral efferent cervical vagal stimulation with activation of

Table 1 *Effect of bilateral and unilateral efferent vagal stimulation respectively on the transpyloric flow and gastric motor activity*

Low stimulation of low threshold fibres. High stimulation of high threshold fibre (For details see text)

	Unilateral		Bilateral	
	Low	High	Low	High
Pylorus				
Contract	2/5	14/14	1/4	15/16
Inhibition	0/5	5/14	0/4	
No effect	3/5	0/14	3/4	1/16
Stomach				
Contract	4/5	14/14	3/4	14/14
Inhibition	0/5	0/14	0/4	
No effect	1/5	0/14	1/4	0/14

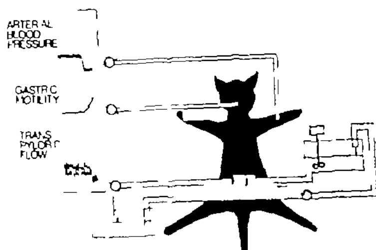


Fig 2 Schematic illustration of experimental setup and preparation of the pylorus in vivo For details see te t

threshold fibres elicited a reduction or cessa- of the transpylonc flow after about 20 s while a d (ithin 4–5 s) contraction was obtained in the mach (Fig. 3 Table 1) In 5 of 14 animals given lateral stimulation of the high threshold fibres re as a small but clear immediate initial in- ase of the transsphincteric flow followed by a uction of the flow I 9 of these cats the gastric ntraction was followed by a prompt decrease in stric tone at the cessation of stimulation There as no difference in the pyloric or gastric response hether the planchnic nerves were cut or left in- ct.

Unilateral vagal stimulation activating the low reshold set of fibres, reduced the transsphincteric ow within 20 s in 2 out of 5 cats. Bilateral stimula- ion activating the low threshold fibres reduced the ranssphincteric flow i only one of 4 animals (Ta- le 1).

Afferent gal nerve timulation The effect of fferent cervical vagal nerve stimulation with the other vagal nerve left intact is seen in Fig. 4 A reduction of the transpyloric flow and a concomit ant gastric relaxation was obtained when the high threshold fibres were activated Activation of the low threshold set of fibres resulted in a pyloric and gastric contraction in a few animals (see Table 2)

Reduction of the transsphincteric flow at afferent nerve stimulation had a delay of 5–10 s while the gastric relaxation always was prompt. In 4 animals, with cut splanchnic nerves, the cut distal cervical vagal nerve was stimulated in afferent direction be- fore and after the contralateral side had been di- vided in the neck. Vagal stimulation elicited a pyloric contraction and gastric relaxation in all 4 animals. The responses disappeared completely af

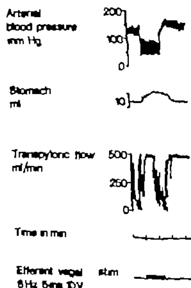


Fig 3 Effect of efferent cervical vagal stimulation on arterial blood pressure gastric motor activity and transpy loric flow Note the initial increase of transpyloric flow before the contraction.

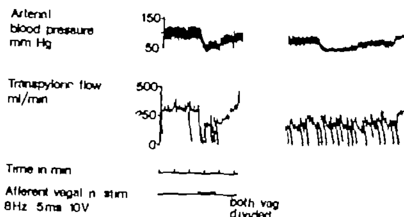


Fig 4 Effect of afferent vagal stimulation on blood pressure and transpyloric flow before (left panel) and after (right panel) dividing of the contralateral cervical vagal nerve

ter the intact vagal nerve had been cut (Fig 4) Efferent stimulation of the cut vagal nerves again induced a contraction of the pylorus

Efferent vagal nerve stimulation during concomitant splanchnic nerve stimulation or noradrenaline-infusion In order to investigate if the vagal nerves also convey relaxatory fibres to the pylorus region the vagal nerves were stimulated when the sphincter tone was increased by a concomitant splanchnic nerve stimulation or local i a infusion of noradrenaline The effect of vagal stimulation during simultaneous splanchnic stimulation was studied in 5 expts A typical expt is illustrated in Fig 5 Splanchnic nerve stimulation with supramaximal currents at 8 Hz elicited a contraction of the pylorus Bilateral efferent vagal stimulation with activation of the high threshold fibres caused [illegible] increased transsphincteric flow

In ? animals the effect of vagal stimulation before and during noradrenaline infusion (0 01-0 02 μg/kg b w /min i.a) was studied The infusion caused contraction of the sphincter Efferent vagal stimulation during concomitant noradrenaline infusion caused an increase of the reduced flow in the animals studied

Table 2 *Effect of unilateral afferent vagal stimulation with the other vagal nerve left intact on the transpyloric flow and gastric motor activity*

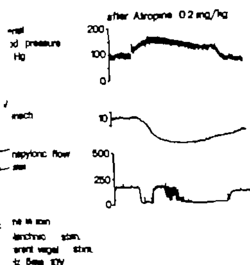
Low stimulation of low threshold fibres High stimulation of high threshold fibres (For detilas see text)

	Low	High
n	5	11
Pylorus		
Contract.	2	11
Inhibition	–	–
No effect	3	–
Stomach		
Contract	1	–
Inhibition	–	10
No effect	4	1

DISCUSSION

In postmortem studies in cats we could confirm the presence of an anatomical pyloric sphincter circular or semilunar in shape (see Fig 1 inset) as earlier described by Torgersen (1942) The vast majority of previous in vivo studies of the pylorus region have been performed by means of recordings of pressure or electrical activity within the pyloric area Pressure recording systems have some disadvantages when gastrointestinal sphincters are investigated It can be difficult to keep the pressure receptor within the spincter region which also might be difficult to exactly identify Furthermore the recording device might interfere with the sphincter activity Recordings with strain gauge force transducers do not allow the study of overall motor function of regions of the gastrointestinal tract This may explain some of the differences in previous studies of the pyloric sphincter

In order to avoid some of these disadvantages and to be able to study the overall motor function of the pylorus region a flow recording method was used Such a method has previously been used in the study of the ileo-cecal sphincter (Pahlm & Ker



: 5 Effect of bilateral efferent vagal stimulation on od pressure gastric motor activity and transpyloric w during concomitant splanchnic nerve stimulation.

ner 1976) and has been shown to have several lvantages. Thus, the interference from a balloon catheter within the sphincter region is avoided id even small variations in sphincter tone can be uded However when perfusion method is used veral factors have to be taken into consideration hus, the perfusion pressure has to be kept at a onstant level by using a reservoir with a large ross-sectional area and recirculation pump The erfusion and drainage tube must have a capacity which is larger than the maximal flow through the phincter and the tubes have to be placed close to he sphincter area in order to avoid secondary ef ects from the canalis-antrum area and the luodenum. As saline was used as perfusion fluid interference due to viscosity could be xcluded The temperature of the perfusion fluid has been shown to be of great importance (Pahlin 1975) Therefore, the temperature of the perfusion fluid was kept around 38°C Great care was taken not to tie the ligature around the proximal tube too tight. At the end of each expt no sign of ischemic injury to this region was observed

Electrical field stimulation of isolated strips of muscles from the pylorus of cat dogs and opossum suggests an inhibitory innervation of the gastroduodenal junction (Anuras et al 1974) Efferent vagal stimulation in dogs, after pretreatment with atropine caused an inhibition of spontaneous pyloric activity indicating an atropine-resistant inhibitory innervation of the pylorus (Mir et al. 1977) In the present expts., efferent vagal stimulation caused an initial relaxation of the pylorus with an increased transphincteric flow followed by a contraction of the sphincter also indicating the presence of relaxatory vagal fibres to this region To further confirm the existence of such fibres within the vagi, these were stimulated during concomitant increase of the sphincter tone as the intestinal tone has been considered to determine which type of intestinal response to vagal stimulation that can be induced (Garry 1957 Van Harn 1963). Thus low tone of the intestine was said to favour excitatory responses and high tone inhibitory responses. Therefore the vagal nerves were stimulated when the sphincter tone was increased by either an intraarterial noradrenaline infusion or a concomitant splanchnic nerve stimulation. Efferent vagal stimu lation caused an increased flow in both these series of experiments indicating the presence of relaxatory fibres within the vagi to the pylorus Thus, the present expts. support the assumption that the vagal nerves convey both excitatory and inhibitory fibres to the pyloric sphincter

The physiological significance of the vago-vagal excitatory reflex to the pylorus is unclear Paintal (1954), Andrew (1957) and Iggo (1957) have hown an increased discharge in vagal afferent fibres on stimulation of either chemo- or mechanoreceptors of the intestine stomach and oesophagus. Harper et al (1959) and Jansson (1969) directly stimulated the afferent fibres within the vagi and found a reflex activation of the vagal relaxatory fibres to the stomach. This gastric response was abolished by vagotomy of the contralateral vagus indicating a vago-vagal relaxatory reflex. In the present study a gastric relaxation at afferent vagal stimulation and a concomitant contraction of the pylorus was always obtained. This reflex response disappeared after bilateral vagotomy indicating the presence of a vago-vagal excitatory reflex to the pylorus. Jansson (1969) suggested that the vago-vagal relaxatory gastric reflex may form part of the normal gastric emptying. It is possible that even the vago-vagal excitatory pyloric reflex may participate in the normal gastric emptying and in the prevention of bile reflux

In the present expts. the latency of the pyloric responses was much shorter at afferent (5–10) than at efferent (20 s) stimulation of the cervical

vagi These rather long latencies might indicate ac tivation of composite neurohumoral mechanisms e g the release of gastrointestinal hormones or simply reflect that the innervation of the sphincter regions is far more complex than of the gut itself (Goyal & Rattan 1975) The faster responses to afferent vagal stimulation might then be due to a more selective neuronal activation through a poly synaptic pathway rather than the crude mixed ef fects on smooth muscle cells intramural ganglia and gut endocrine cells elicited at efferent stimula tion

It has previously been shown that the vagal nerves contain at least 2 groups of efferent nerve fibres which control the gastric motor activity (Martinson & Muren 1963 Martinson 1965*a*) Thus excitatory fibres could be selectively activated when comparatively low threshold parameters were used If the duration of the stimulatory pulses was increased to high threshold values vagal relaxatory fibres were activated as well The high threshold fiber group also include fibres stimulating the acid and pepsinogen secretion in the stomach as well as vasodilatatory fibres (Martinson 1965*b*) In the present study contractions were regularly obtained when stimulating parameters activating the high threshold fibres were used e g the same parameters which activate the non-cholinergic non-adrenergic relaxatory fibres to the stomach This vagal re sponse with pyloric contraction and gastric relaxa tion acid and pepsinogen secretion and vasodilata tion seems all suitable for the digestive gastric activity

Supported by the Swedish Medical Research Council (grant no 17X-05220) and by Göteborgs Läkaresällskap

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n note on the bolus injection residue detection method ır measurement of capillary permeability

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e bolus injection external registration method r measurement of capillary extraction was ntro- iced by Sejrsen in 1970 An elaboration of this ethod and further experimental support has en given in a later article (Sejrsen 1979). In a note this method Garby & Gronlund (1978) perform a eoretical analysis on the basis of some conditions d assumptions of the method. The present note ms at correcting the errors introduced by Garby & nlund (1978) in their analysis in order to show e irrelevance of the analysis performed

From the article presenting the bolus injection, sidue detection method for measurement of capil- ry extraction (Sejrsen 1970) the following quota- on is taken: A large fraction of the bolus of romium-51 EDTA will pass through the muscle clusively as very fast vascular transit and raction E, will be xtracted over the capillary embrane and thus ha e also an extravascular nsit. By this sentence it was made explicit that e extracted fraction, E of the intra-arterially in- cted indicator bolus has both an intra- and an tra-vascular transit, while the complementary action denoted the transmitted fraction, T does ot permeate the capillary membrane and conse ently has only vascular transit through the ssue.

In the note be Garby & Gronlund (1978) equa- on (11), it is erroneously reproduced from the ar icle by Sejrsen (1970) that the registered curve upplemented by a retropolation from the time here the vascular transit is f ished is an expres- ion of the washout of the extracted fraction located clusively in the interstitial space. This part of the ndicator bolus cannot be measured separately by residue detection which must register the total mount in the area under study given as a relative measure. This implies that their equation (14) also ecomes wrong

From an estimate of the volume of arterial vessels and the actual blood flow level it can be calculated that more than 10 % of the indicator molecules must be present in the arterial vessels at time of maximum height of the curve in the actual experi- ments (Sejrsen 1970 1979) This part of the indi- cator will be separated in an E and T fraction later on in the following part of the washout But these cond tions do not involve that a separation of the E and T fraction is impossible by the retropolation proced re These considerations then imply that equations (9) (10) (1) and (13) in the article by Garby & Gronlund (1978) become unnecessary de mands

For the applicability of the method the essential question is how accurately the registered residue curve can be resolved in the E fraction which enters the interstitial space, and the T fraction with an exclusively intravascular transit. Due to the consid- erable difference in transit times of the extracted and the transmitted fractions resolution can be per formed with good approximation In average a fac tor of 26 has been found between the values of rate constants of the two types of transit within the first minutes of the washout curves for chromium-51 EDTA in skeletal muscle Even for the fastest part of the transit of the extracted molecules compared to that of the slowest part of the transit of the transmitted molecules there is a difference in rate constants of at least a factor of 7 (Sejrsen 1979) It is important to notice that the time interval for the retropolation is relatively short. It is assumed that the tissue to blood exchange of indicator in the retropolation interval has the same washout kinet ics as the curve part used for the retropolation It is observed that the fractional washout rate is almost constant for the curve part employed This is pre sumably effected by diffusional mixing of the indi- cator in that part of the interstitial compartment

positioned in close relation to the capillary wall which constitutes a pronounced diffusional resistance for the actual molecules

By residue detection the total amount of indicator in the area under study will contribute to the registered counting activity When the total input to the system has a shorter duration than the shortest transit time of the system (Sejrsen 1969) then the maximum height of the registered washout curve is a relative measure of the total bolus input The washout of the extracted fraction from the total system is expressed relatively by the registered curve after some time when the vascular transit of the transmitted fraction for all practical purposes has come to an end This curve part can be simulated by some mathematical expression employed for the retropolation of the curve The value of this curve at the time of maximum height of the registered curve yields the extraction fraction when expressed relative to the maximum value of the registered curve As the total amount of indicator is present in the area under study at this time the total amount of the extracted fraction is also present at that time The location of the extracted fraction will at that time be partly in the different segments of the vascular bed and partly in the interstitial space There is no demand of complete spatial separation of the extracted and the transmitted fraction at that time and in principle no demand concerning an insignificant back diffusion from interstitial to vascular space

Subtraction of the retropolated curve from the registered curve gives the transit curve for the transmitted fraction through the vascular system expressed as a relative measure The vascular volume (calculated from the mean transit time of an intravascular indicator and the directly measured blood flow in separate experiments) divided by the mean transit time of this vascular transit curve yields the value of the blood flow This value can be compared to the directly measured blood flow The configuration of the intravascular transit curve can be compared to that of an intravascular indicator These comparisons have been performed in order to investigate the validity of the suggested retropolation procedure (Sejrsen 1979) The conditions and assumptions necessary for the method are listed and discussed in detail in the article by Sejrsen (1979)

A correction of the erroneous propositions introduced by Garby & Grønlund (1978) has the effect that all the supplementary conditions and assumptions proposed by them do not constitute requirements for the method One might speculate that their approach could be used as basis for an alternative analysis but the unrealistic demand that the E and T fractions are separated at the time of maximum of the registered curve eliminates such possibility

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...uction of adrenaline turnover in cardiovascular ...as of rat medulla oblongata by clonidine

...L FUXE, GÖSTA JONSSON PER BOLME, KURT ANDERSSON LUIGI F AGNATI
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...as recently been postulated that clonidine is an ...naline (A) agonist in the central nervous system ... that this action could be of importance for its ...odepressor activity (Fuxe et al 1976, 1979) ...refore, in the present paper the acute effect of ...idine in clinically relevant doses have been ...died on noradrenaline (NA) and adrenaline ...over in the hypothalamus and in the dorsal ...line area of the caudal medulla oblongata ...DMO), using inhibitors of dopamine β-hy...xylase, FLA 63 (bis- (4-methyl-1-homo...erazinyl-thiocarbonyl)-disulphide) and of ...nylethanolamine N-methyltransferase SK&F ...139 (7,8-dichloro-1,2,3,4-tetrahydroisoquinoline ...drochloride) (Corrodi et al 1970 Pendleton ...al. 1976).

Male specific pathogen free Sprague-Dawley rats ...ve been used (150 g b w). The rats were kept 5 ... r cage under regular light–dark conditions (light ... h at 6 a.m. and off ... 6 p m). They were give... ...od pellets and water ad libitum. They were killed ... decapitation between 9 and 11 a.m. Clonidine ...s given into the jugular vein under light ether ...esthesia. Immediately before the clonidine injec...on FLA 63 or SK&F 64139 were given in doses of ... mg/kg (p) and 40 mg/kg (p) respectively 1 or ...h before killing.

By means of a rectangular punch (3 4 mm) the ...ypothalamus was dissected out from frozen ...oronal section of the diencephalon. The DCMO ...as also dissected out by means of a rectangular ...unch (1×3 mm) from frozen coronal 1 mm thick ...ection of the medulla oblongata, the caudal plane ...eing located immediately behind the obex. DCMO ...ontains, after alia, the nucleus tractus solitarius ...ucleus dorsalis motorius nervi vagi and parts of the ...ucleus commissuralis (see Fuxe et al 1979). The ...atecholamine levels were determined by means of ...igh pressure liquid chromatography in combina...tion with electrochemical detection, using α-methyl dopamine as an internal standard (Keller et al. 1976 Medford et al. 1977)

Clonidine in a dose of 30 μg/kg significantly reduced the SK&F 64139 induced A depletion in the DCMO (Fig. 1). An i v injection of clonidine in a dose of 7.5 μg/kg significantly reduced the FLA 63 induced A depletion in the DCMO while the FLA 63 induced NA depletion in this area was uninfluenced (Table 1). In the hypothalamus however

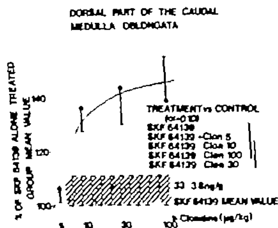


Fig. 1. The effects of clonidine on the SK&F 64139 induced A depletion in the dorsal midline area of the caudal medulla oblongata (DCMO). For treatment, see text. The values are expressed in per cent of SK&F 64139 untreated group mean value. Means ±S.E. are shown out of 3 rats. The hatched area represents the S E. of the SK&F 64139 alone group mean value. The A levels in the untreated group was 60±6 ng/g tissue (means ± S E.). Thus, 2 h of treatment with SK&F 64139 (40 mg/kg) produced approximately 50% depletion of the A stories in the DCMO (33±3 ng/g tissue). The statistical analysis is given in the table of the figure. The Dunn test was used with an experimental wise error rate of ≈0 1. The groups not embraced by the same line are significantly different.

Table 1 Effects of clonidine on FLA 63 induced changes in CA stores in dorsal midline area of the medulla oblongata (DCMO) and hypothalamus

Treatment	Dose of clonidine (μg/kg)	DCMO NA % (ng/g tissue)	DCMO A % (ng/g tissue)	DCMO DA % (ng/g tissue)	Hypothalamus NA % (ng/g tissue)
Saline		100±8 (5)	100± (5)	100±3 (5)	[illegible]
		(4 668±354)	(104±23)	(396±17)	[illegible]
Clonidine h	10	89± (5)	1_5±11 (5)	102±4 (5)	[illegible]
Clonidine ? h	30	94±9 (5)	116±13 (5)	105±6 (5)	106±3 (5)
FLA 63 1 h		100±6 (4)	100±7 (3)	100±10 (4)	[illegible]
		(3 064± 15)	(79±5)	(1 148±77)	[illegible]
Clonidine + FLA 63 1 h		109±6 (8)	134±17 (7)	147±10 (8)	[illegible]
FLA 63 2 h		100±1 (5)	100±15 (5)	100±11 (5)	[illegible]
		(? 653±317)	(47±7)	(958±107)	[illegible]
Clonidine + FLA 63 2 h	7 5	100±11 (8)	123±21 (8)	99±13 (8)	120±11 (8)

For treatment, see text Means ± S E are shown The values are given in per cent of untreated group mean value of FLA 63 1 h group mean value and of FLA 63 2 h group mean value respectively The absolute levels are given in parentheses [illegible] tissue As seen from the absolute levels, h of treatment with FLA 63 reduces noradrenaline contents and [illegible] contents in the DCMO to 56 % and 45 % respectively of untreated group mean value In the hypothalamus [illegible] edu e noradrenal ne and adrenaline levels to 38 % and 6_ % respectively of untreated group mean value Number of [illegible] parenthesis Statistical analysis was made by means of Mann-Whitney U-test $P<0.05$ <0.01

clonidine in the same dose instead significantly reduced the FLA 63 induced NA depletion without influencing the FLA 63 induced A depletion in this area (Table 1)

The present findings give evidence that clonidine administered into the jugular vein can reduce A turnover in the DCMO since clonidine reduced both the FLA 63 and the SK&F 64139 induced A depletion in the DCMO in doses from 7.5 to 30 μg/kg However no evidence was obtained that A turnover was reduced in the hypothalamus by 7 5 μg/kg of clonidine These results are in agreement with recent reports that clonidine can reduce A turnover in brain (Fuxe et al 1979*b* Scatton et al 1979) The present results therefore support our hypothesis that clonidine at least in the DCMO can be an A agonist in clinically relevant doses It is known that injections into the jugular vein of clonidine in the doses used produce vasodepressor actions (Bolme et al 1974) It is unclear however if the reduction of A turnover observed i due to activation of post or pre synaptic α component of central A receptors

It is of interest that hypothalamic NA was more responsive to clonidine treatment than hypothalamic A Thus clonidine in a dose of 7.5 μg/kg seemed to reduced hypothalamic NA turnover since it significantly reduced the FLA [illegible] induced NA depletion in the hypothalamus The[illegible] fore when discussing the mechanism of action [illegible] the cardiovascular effects of clonidine both [illegible] NA and A mechanisms must be considered A[illegible] aline mechanisms in the medulla oblongata [illegible] NA mechanisms in the hypothalamu seem to [illegible] a similar sensitivity to clonidine In conclusion the present paper gives evidence that clonidine in doses from 7 5 μg/kg can reduce A turnover in the DC[illegible] and NA turnover in the hypothalamus

This work was supported by a grand (04X-4626) from the Swedish Medical Research Council Clonidine [illegible] generously supplied by Boehringer Ingelheim (Germany)

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(tissue)	DA (ng/g tissue)
(5)	100 11 (5)
	(697±79)
(5)	103 10 (5)
(5)	104±7 (5)
(4)	100± (4)
	(990+26)
(8)	114_8 (8)
(5)	100±7 (5)
	(788 60)
(10)	108±6 (10)

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FLA 63 2 h		100±1 (5) (? 653±317)	100±15 (5) (47±7)	100±11 (5) (958±107)	100 11(5) (1 ?27 1?)
Clonidine + FLA 63 2 h	7 5	100±11 (8)	1_3±21 (8)	99±13 (8)	120±11(8)

For treatment see text Means ± S E are shown The values are given in per cent of untreated group mean the of F 1 h group mean value and of FLA 63 ? h group mean value respectively The absolute levels are given in parenthe tissue As seen from the absolute levels ? h of treatment with FLA 63 reduces noradrenaline contents and the contents in the DCMO to 56% and 45% respectively of untreated group mean value In the hypothalamus the tre duces norad enaline and adrenaline levels to 38% and 6_% respectively of untreated group mean value Number of parenthesis Statistical analysis was made by means of Mann-Whitney U test $P<0\ 05$ $<0\ 01$

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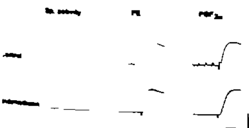
ıe influence of prostaglandin synthetase inhibition ı the spontaneous contractile activity ıd induced responses of the human oviduct

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e isthmic portion of the human Fallopian tube mprises three muscle layers with different fibre ection the outer longitudinal the intermediate cular and the innermost longitudinal, the latter appearing towards the distal end of the isthmus vid & Czernobilsky 1968 Daniel et al 1975) We e recently demonstrated that in the outer longitudinal layer at the distal end of the isthmus, i.e. e ampullary isthmic junction (AIJ) there is a minance of α-mediated excitatory response after posure to noradrenalin adrenalin, phenylephrine transmural nerve stimulation (Lindblom et al 79) Moreover prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) has en show to be a potent stimulator of the longitudinal smooth muscle in this region (Lindblom et l. 1978)

The present report concerns the influence of indomethacin an inhibitor of PG ynthesis on spontaneous activity and α-adrenoceptor responses of longitudinal smooth muscle at the AIJ of the human oviduct.



g 1 Left panel Spontaneous contractile activity (upper tracing) and the effect of indomethacin (40 μg/ml) lower tracing) on the spontaneous contractile activity of ongitudinal musculature from the human oviductal isthmus Middle panel The stimulatory effect of phenylephrine (10 μM) (upper tracing) and the effect of phenylephrine after total inhibition of spontaneous activity induced by indomethacin (40 μg/ml) (lower tracing). Right panel The stimulatory effect of $PGF_{2\alpha}$ (0.1 μg/ml) (upper tracing) and the effect of $PGF_{2\alpha}$ after total inhibition of spontaneous activity induced by indomethacin (40 μg/ml) (lower tracing) Vertical calibration = 4 mN horizontal calibration 3 min.

Tissues from 20 women 25–4. years of age were included in the study All patient had regular menstrual cycles and hormonal contraception had been withdrawn for at least months prior to bilateral tubal resection. Routine premedication and general anesthesia were given to all patients. A 4–5 cm long portion of the tube was excised without clamping and immediately placed in chilled oxygenated Krebs-Ringer bicarbonate buffer The particular phase of the menstrual cycle was estimated by serum levels of progesterone and estradiol-17β Muscle strips from the longitudinal layer of the isthmus were dissected under a stereo microscope

The strips were approximately 4 mm long, with a cross-sectional area of approx. 1 mm² The muscle strips were suspended in 50 ml organ baths containing Krebs-Ringer buffer at a temperature of 37°C and aerated continuously with 5% CO_2 in O_2 One end of the strip was tied to a tissue holder by a silk ligature and the other end was connected to an isometric force transducer by a ligature

The contractile activity was recorded under passive tension of 4 mN (for further methodological details, see Lindblom et al. 1979). The drugs used were Phenylephrine hydrochloride (Sigma Chemicals Company Mo USA) $PGF_{2\alpha}$ (tromethamine salt kindly supplied by the Upjohn Company Kalamazoo Mich) Phenoxybenzamine hydrochloride (Dibenyline S K & F Laboratories Ltd. U K) and indomethacin (kindly supplied by Merck Sharp & Dohme International, Rahway N.J) Phenylephrine and $PGF_{2\alpha}$ were dissolved in

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Key ords Smooth muscle oviduct, human prostaglandins, adrenoceptors, calcium.

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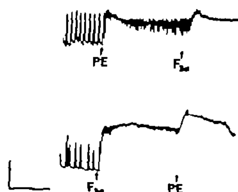


Fig 2 The effect of $PGF_{2\alpha}$ 1 μg/ml on longitudinal muscle maximally contracted by phenylephrine 10 μM (PE) (upper record) and the effect of phenylephrine 10 μM on a similar preparation maximally contracted by $PGF_{2\alpha}$ 1 μg/ml (lower record) Vertical calibration = 4 mN horizontal calibration = *5 min*

buffer whereas indomethacin was dissolved in 0 9% sodium chloride with 0 05% Tween 80

Some 10–30 min after the mounting of the specimens in the organ chambers spontaneous contractile activity appeared Addition of indomethacin to the bath in a final concentration of 10–40 μg/ml caused a concentration-dependent reversible inhibition of the spontaneous contractile activity The inhibitory effect appeared within 2–5 min after the addition of the drug to the bath Total inhibition of the spontaneous activity was achieved at 30–40 μg/ml (Fig 1) The inhibitory effect lasted for approximately 15 min Phenylephrine (10 μM) caused a marked stimulatory effect on the preparations characterised by a tetanic contracture After total inhibition of spontaneous activity induced by indomethacin the effect of exogenous PE was unaltered (Fig. 1) The excitatory effect of $PGF_{2\alpha}$ (0 a μg/ml) was similar to that of PE and also this response was unaffected by indomethacin (Fig 1) Maximum contraction evoked by PE could be further augmented by $PGF_{2\alpha}$ Similarly preparations maximally contracted by $PGF_{2\alpha}$ exhibited a further increase in tone after the addition of PE (Fig 2) After α adrenoceptor blockade with phenoxybenzamine (0 1 μM) the effect of PE was abolished whereas the response of $PGF_{2\alpha}$ was unaffected In nominally calcium-free solution both the spontaneous activity as well as the responses to $PGF_{2\alpha}$ or PE were abolished

Both adrenergic nerves and local hormones such as prostaglandins (PGs) have been claimed to be of crucial importance in the regulation of oviductal contractility which in turn is considered to influe[illegible] the transport of the ovum through the tube [illegible] review see Aref & Hafez 1973). Studies on [illegible] uterine tissues from the pregnant rat have [illegible] indicated that an intact PG synthesis is essential [illegible] spontaneous myometrial contractility [illegible] (Aiken 1974) In the latter study indomethacin [illegible] concentration of 0 01–3 0 μg/ml was found to [illegible] hibit spontaneous contractions and high concentra[illegible] tions also abolished the stimulatory effect of the P[illegible] precursor arachidonic acid On the other hand [illegible] der et al (1977) failed to demonstrate an [illegible] effect of indomethacin on human oviductal [illegible] in vitro and concluded that PGs are not d[illegible] involved in the regulation of myogenic activity [illegible] this tissue The very poor spontaneous activity [illegible] the large tubal segments used in that study [illegible] doubt however as to the validity of their conclu[illegible]sions

The present investigation performed on [illegible] strips from the longitudinal musculature of the hu[illegible]man oviductal isthmus demonstrates that [illegible] domethacin in concentrations between 10–40 μg [illegible] inhibits spontaneous contractile activity [illegible] versible and concentration-dependent [illegible] Moreover the initial contractile pattern can be [illegible] stored by the addition of small amounts of PGF [illegible] Together these results suggest that PG synthes[illegible] of crucial importance for spontaneous activity [illegible] the human oviduct in vitro Both PE and PGF [illegible] elicit powerful excitatory responses and these [illegible]fects are not reduced by indomethacin at concentra[illegible]tions that cause total inhibition of spontaneous [illegible]tivity These observations demonstrate that the [illegible]hibitory effects of indomethacin are not unspec[illegible] in nature and that α-adrenoceptor mediated co[illegible]traction is not mediated via an influence on P[illegible] synthesis indicating that the sympathetic nerv[illegible] may utilize other paths in the activation of the [illegible]cle cells This suggestion is strengthened by [illegible] finding that after maximum contraction evoked [illegible] $PGF_{2\alpha}$ a further increase in tension could [illegible] achieved by the addition of PE and vice vers[illegible] Nevertheless the excitatory actions of PGs [illegible] α-adrenoceptor stimulation certainly invol[illegible] common final mechanism since both effects [illegible] abolished in nominally calcium-free solution.

This investigation was supported by grants from Knut [illegible] Alice Wallenberg Foundation The Swedish Medical [illegible]search Council (2873) and The Göteborg Medical Soc[illegible] Sweden.

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n the VIP-ergic innervation of the feline pylorus

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noactive intestinal polypeptide (VIP), isolated and sequenced by Said and Mutt (Said & Mutt 1970, utt & Said 1974) ha been localized by immunohistochemistry to peripheral neurons in var us nerves (Lundberg et al 1978) and organs, i.e the gastrointestinal region (Larsson et al 1976) urthermore VIP ha been localized to sphincter gions (Alumets et al. 1979) and found to relax oth the antral smooth muscle (Morgan et l 1978) nd the cardia sphincter (Rattan et al 1977) In ddition, VIP can be released from the gut by lectrical stimulation of the vagal nerves in the pig Fahrenkrug et al. 1978)

It was also recently demonstrated that close i.a nfusions of this peptide caused a gastric relaxation nd an increased blood flow to the stomach (Ek und et al 1979), a response similar to the vagally nduced effect at high threshold stimulation Martinsson 1965) Together these find ngs strong ly suggest that VIP may be one of the neurotransmitters in the gastrointestinal tract (see Fahrenkrug et al 1978 b) The purpose of the present study wa to demonstrate VIP-ergic neurons within the feline pylorus by immunohistochemistry and to further study the simultaneous effects on gastric and pyloric motility when VIP was injected in local gastric artery

The immunohistochemical studies were performed as described previously (for references, see Edin et al 1979b), using VIP antiserum 5603-5 (Fahrenkrug et al 1977 1978) Gastric motility as studied by volume recordings of flaccid intragastric balloon at constant intraluminal pressure (Martinsson 1965) Pyloric motility was recorded a changes of an applied constant flow of body warm saline acros the sphincter (Edin et al 1979). 6 cats (2.5–3 kg) of either sex in chloralose anesthesia (50 mg/kg) were used. A fine heparinized plastic catheter (1 mm) was introduced along the greater curve of the tomach via the splenic artery in retrograde direction In its final position ink infusion after the experiments showed distribution to the first part of the duodenum the pylorus and the stomach VIP (kindly donated by Prof Viktor Mutt Karolinska Institutet Stockholm) was dissolved in saline and given as single bolus doses (0 03–1.3 nmol) via this catheter The VIP injections induced a prompt gastric relaxation and a marked increase of the transpyloric flow at all doses gi en but only in 50% of the experiment with the lowest dose (0 03 nmol) The effects lasted about 10–15 min and 4–5 min respectively (Fig. 1)

The VIP-containing neurons within the pylorus had characteristic d stribution pattern. VIP immunoreactive nerve cells were observed in moderate numbers in the myenteric and high numbers in the submucous plexus. Numerous VIP immunoreactive fibres were seen in the lamina propria, in the circular smooth muscle layer (Fig.) and in the myenteric plexus.

In the stomach VIP-immunoreactive nerve cells were observed both i the myenteric plexus (fewer than in pylorus) and in the submucous plexus (as in pylorus) In the corpus-antrum the circular muscle layer contained a medium dense network of nerve fibres the lamina propria mucosae a dense network, and the longitudinal muscle layer few single nerve fibres.

I a previous study we have demonstrated enkephalin immunoreactive nerve cells and fibres mainly in the myenteric plexus and the circular smooth muscle layer of the pylorus (Edin et al 1979b). Furthermore enkephalin as well as vagal stimulation caused a pyloric contraction effects which were blocked by naloxone (Edin et al 1979b). The VIP immunoreactive fibres in the circular muscle layer had a distribution very similar to the enkephalin nerves. Nevertheless the two peptides have antagonistic effects since VIP causes pyloric relaxation It is interesting to note that, in

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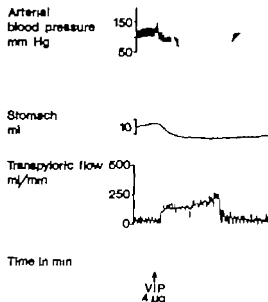


Fig 1 Recordings of intraarterial blood pressure gastric motility and transpyloric flow in the cat at i.a bolus injection of VIP (1.3 nmol) Note the long-lasting relaxation of the stomach (10–15 min) and the more short-lasting dilatation of the pylorus (4–5 min)

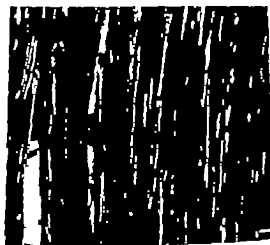


Fig 2 Immunofluorescence micrograph of the circ. smooth muscle layer of the feline pylorus A less network of longitudinally sectioned VIP immunoreact. nerve fibres is seen Bar indicates 50 µm

agreement with the results of Eklund et al (1979) the VIP induced receptive relaxation has a very long duration whereas the pyloric relaxation seen in this study was more short lasting It seems therefore possible that neuropeptides may play important roles in the regulation of the pyloric sphincter tonus

The technical assistance of Mrs Waldtraut Hiort and Mrs Harriet Andersson is gratefully acknowledged This study was supported by the Swedish Medical Research Council (grants nos 17X 5220 04X 2887 04X 2207 and 04P-4173) by the Medical Faculty University of Göteborg Göteborgs Läkaresällskap and Harald och Greta Jeanssons Stiftelse Sweden

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3 to 2 coupling of the Na–K pump responsible r the transepithelial Na transport in frog skin sclosed by the effect of Ba

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cording to the two-membrane hypothesis oefoed-Johnsen & Ussing 1958) one would ex "t an increase in the K permeability of the out rd facing membrane to result in an active out rd transport of K (Fig. 4) Polyene antibiotics rease the permeability of cell membranes for K d other ions (De Kruijff & Demel 1974) Addition polyene antibiotics (filipin and amphotericin B) to outside bathing solution of the isolated frog k n l in fact result in an active outward transport of (Nielsen 1971 Nielsen 1972) In a recent pape ielsen 1979) it is shown that the filipin-induced tive outward K transport is coupled t the active ad N transport and the data suggest that the upling ratio between the act ve transepithelial and K transport is 1 5 (3Na/2K) If the act ve a transport acros the isolated frog skin is carried out by a Na–K pump with a coupling ratio of 1.5 then the addition of a component which completely inhibits the passive K current across the inward facing membrane should i the absence of filipin initially result in 66.6% inh bition of the hort-cir cuit current (SCC) (see below)

The experiments were performed on male and female frogs (*R na t mporana*) as described previ- ously (Nielsen 1977) The epithelia were dissected from collagenase treated skins (Johnsen & Nielsen 1978) and mounted in perspex chambers (area 0 7 cm²) and bathed in stirred Ringer s solution (Na 115 K 2.5 Ca² 1 Mg² 1 Cl 117 CO²₃ .5 PO³⁻ 1 glucose 5 mM pH 7.8)

Addition of 5 mM Ba to the inside bathing solution (IBS) resulted in an inh bition of the SCC and in an increase in the transepithelial resistance (Fig. 1) About 5 min after the addition of Ba the SCC started to recover and the resistance started to decrease The recovery phase was com- pleted 70–90 min after the addition of Ba The recovery varied from 30% to complete recovery In the present paper only the initial effect of Ba will be discu sed When Ba is added to the IBS of isolated epithelia the same result is obtained as in whole skins (Fig. 2) but the inhibition is faster because the thicknes of the unstirred layer on the inside has been reduced by removal of the con- nective tis ue

The primary i hibitory effect of Ba is much faster than the secondary effect (the recovery) of Ba so the two effect are separated in time Therefore it is possible to measure the Ba in- duced initial inh bition without getti g interference from the subsequent recovery The i tial Ba in- duced inh bition is plotted agai st the Ba con- centration in the IBS (Fig. 3), the Ba induced inh bition of the SCC increases with increasing Ba concentration until a maximum inhibition is reached Addition of Ba (5 mM) to the IBS a

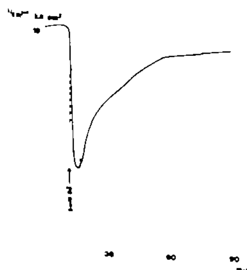


Fig. 1 Effect of Ba on the short-circuit current across isolated frog skin At the arrow Ba (5 mM) is added to the inside bathing solution. — Short-circuit current μA/cm² — transepithelial resistance KΩ cm²

ctroneutrality in the cells the net Na flux acros outward facing membrane has to be reduced by amount of K which is pumped into the cells. If coupling ratio for the Na–K pump is 1.5 a mplete blocking of the K flux via the K channel ll initially result in a 66 6% inhibition of the SCC ereafter the SCC will change probably because presence of Ba causes secondary increase in K permeability of the membranes as show in rdiac muscle (Hermsmeyer & Sperelakis 1970) e observations that maximum Ba -induced ini- l inhibition of the SCC is not significantly differ t from 66 6% (Fig 3) supports the idea that the nsepithelial Na transport in frog skin is carried t by a Na–K pump with a coupling ratio of 1.5 In mmetrical cells (such as erythrocytes, muscle d nerve) it is generally accepted that the coupling tio for the Na–K pump is 1.5 (Thomas 1972)

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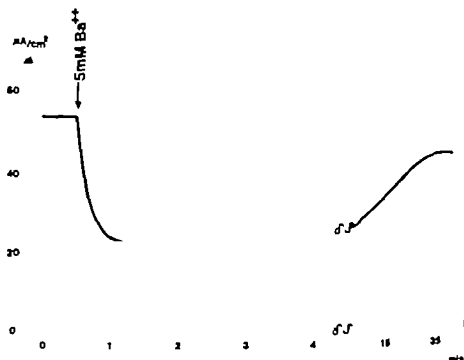


Fig 2 Effect of Ba on the short-circuit current across an isolated epithelium

concentration which gave maximum inhibition resulted in a 65 0±1 9% (n=10) inhibition of the SCC this inhibition is not significantly different from 66 6% Ba has been shown to reduce the K conductance in frog heart (Hermsmeyer & Sperelakis 1970) in frog muscle (Henderson 1974) and in frog skin (Nagel 1978 Nielsen 1979)

According to the two-membrane hypothesis (Koefoed-Johnsen & Ussing 1958) the active Na transport across the isolated frog skin occurs in two steps passive diffusion across the outward facing membrane of the epithelium followed by an act extrusion across the inward facing membrane the Na–K pump If the active Na transport is car ried out by a Na–K pump with a coupling rat 1 5 then according to this model (Fig 4) of the SCC across the inward facing membrane is car by K ions and by Na ions Addition of a comp nent (Ba) which completely blocks the K chan would abolish the K current via the K channel the K which is pumped into the cell via the Na pump cannot leave the cells In order to

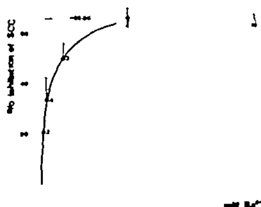


Fig 3 Maximum percentage reduction of the short-circuit current elicited by various concentrations of Ba Values are the mean ± S E. figures at the curve are number of expts.

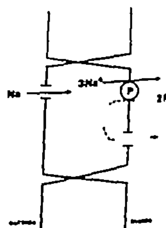


Fig 4 The two-membrane hypothesis. P Na–K pump with a coupling ratio of 1.5 (3Na/2K)

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hloride transport by self exchange and by KCl salt iffusion in gramicidin treated human red blood cells

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CASS, A. & DALMARK M Chloride transport by self-exchange and by KCl salt diffusion in gramicidin-treated human red blood cells. Acta Physiol Scand 1979 107 193–203 Received 10 Jan. 1979 ISSN 0001-6772 Department of Biophysics University of Copenhagen, Panum Institute and the Department of Medicine M Frederiksberg Hospital Denmark

The permeability of gramicidin-treated human red blood cell membranes to K and Cl^- has been measured at normal ionic strength (1) by tracer exchange at steady-state distribution of salt, and (2) by net transport of salt in the presence of salt concentration gradient. Under both conditions KCl was the only inorganic salt in cells and medium. In the studies of self-exchanges the electrical driving force on the ions was zero Calculation of permeability coefficients from net salt transport was simplified because the xperiment was designed as special case of the Nernst Planck diffusion regime I the single salt case. Gramicidin altered the cell membranes from being anion to become cation selective Gramicidin increased the potassium exchange without affecting the chloride exchange measurably The chloride exchange showed saturation kinetics as does chloride exchange in normal cells. The net transport of KCl in the presence of constant concentration gradient increased to constant value with increasing gramicidin concentration. At high gramicidin concentrations (0°C pH 7.2) the chloride permeability coefficient calculated from tracer exchange (1.9 10^{-4} cm/s) was 290 times the chloride permeability coefficient calculated from net salt transport (0.65 10^{-6} cm/s) The latter value corresponds to chloride conductance of 4.2 10^{-6} ohm cm^{-2} The chloride permeability coefficient was 1 10^{-6} cm/s at 25°C (pH 6.8) indicating value of 3 for the Q_{10} It appears that normal red cells are anion selective in the sense that anion permeability exceeds cation permeability with factor of more than hundred between 0°C and body temperature The anion exchange, e. the Hamburger shift, is tightly coupled transport process which is several orders of magnitude faster than anion transport by salt diffusion.

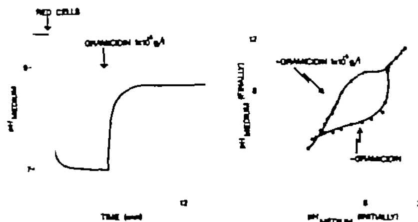
Key ords Chloride transport red blood cells gramicidin

t was suggested by Warburg (1922) and Van Slyke t al. (1923) that the human red blood cell is selectively anion permeable in the sense that anion permeability exceeds cation permeability by orders of magnitude The electroneutrality condition dictates that chloride transport is limited to exchange for other anions like chloride (Chloride self-exchange) or bicarbonate (the Hamburger shift) because the cation permeability is low However it is impossible from studies in normal cells to decide whether (1) all the anions are transported by independent migration, or whether (2) greater or smaller fraction of anion efflux and influx is linked together as an electroneutral—chemically coupled—anion exchange The latter alternative is favoured by the characteristics of chloride self-exchange which shows features of facilitated diffusion (reviewed by Dalmark 1976). The low electrical membrane conductance relative to the chloride tracer exchange in the red cell of the giant salamander Amphiuma, is consistent with this view (Lassen et al. 1974 1978)

The cation permeability of red cell membranes is increased by addition of various ionophores. In these modified cells the electroneutrality restriction is fulfilled when the choride transport takes place as

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1 The pH-shifts of low ionic strength medium following suspension of red cells in the medium and, later on, after ...tion of gramicidin to the cell suspension (Fig. 1). Acidification and alkalization of low ionic strength media ...wing suspension of red cells in the media (hematocrit 0.01 v/v) as functions of the pHs in the media prior to the ...ension of the cells in absence (●) or in presence (○) of gramicidin (10^{-4} g/l) (Fig. 1*b*). The cells were washed in salt ...tion (150 mM KCl, 27 mM sucrose) and titrated to pH 6.6 (38°C) before the cells were suspended in the low ...strength media (1 mM KCl, 302 mM sucrose) at 38°C. The pH of the suspending medium was adjusted with KOH ...Cl before the cells were suspended.

...rmined either ( ) by coulometric titration (CMT ...ride titrator Radiometer Copenhagen, Denmark), or from the distribution of ^{36}Cl between cell water and ...ium together with the chloride concentration in ...ium. The potassium content in the cells was deter... ...ed either ( ) by flame photometry (Radiometer ...penhagen, Denmark), or (*b*) from the potassium con... ...tration in medium and the distribution of ^{42}K. The ...ssium and chloride concentration in medium was de... ...ined by flame photometry and coulometric titration. ...e equation of the permeability coefficient employed in ...transport experiments is derived in Results.

...ESULTS

...e results will be presented in 4 sections. The first ...ction describes the effect of gramicidin on ionic ...lectivity of the red cell membrane at low ionic ...rength. The second and third section contain ex... ...rimental data on ( ) potassium and chloride self ...change, and (*b*) net transport of potassium ...loride and water at normal ionic strength. The ...st section contains an analysis of the net transport ...periments and the estimate of the chloride per... ...eability coefficient.

...ffect of gramicidin on ...electivity

...rapid acidification of the medium occurs when ...ormal red cells are suspended in low ionic strength ...edia at neutral pH (Davson 1939, Wilbrandt 1940).

It is generally believed that anions like chloride and hydroxyl ions rapidly reach thermodynamic equilibrium. The distribution of chloride across the untreated membrane determines the pH of an unbuffered KCl solution because the cellular pH is constant owing to the high buffer capacity of hemoglobin. Fig. 1 shows the acidification of a low ionic strength medium following suspension of red cells in the medium. Subsequent addition of gramicidin to the cell suspension shifts the pH in the alkaline direction. Although the mechanism of the acidification is debated the alkalization of the medium following addition of gramicidin appears to be a transport of protons because proton permeability is greatly increased in gramicidin-treated thin lipid membranes (Myers & Haydon 1972). The potassium diffusion potential in gramicidin-treated red cells determines the distribution of protons across the cell membranes in the experimental situation employed.

Fig. 1*b* (filled circles) demonstrates the acidification of the medium as function of the pH prior to normal cells were suspended in an unbuffered low ionic strength medium: the change in pH increased with the pH initially present in the medium. N... change in pH was observed at an initial pH of 4.6. This pH value was predictable from the chloride distribution across the cell membranes the activity

(1) an anion exchange or (2) a *net anion transport* accompanied by cations (net salt transport) in the presence of a *concentration* gradient The effect of ionophores on net salt transport has been interpreted *as if* the anion transport by salt *diffusion was* much slower than anion transport by self-exchange (Harris & *Pressman 1967* Scarpa et al 1970 Tosteson et al 1973 Wieth et al 1973 Hunter 1977 Parker et al 1977 Knauf et al 1977) Estimates of the chloride permeability *coefficient* were obtained by using the Nerst Planck formalism with the Goldman constant field assumption or by flux ratio *measurements* The absence of an inhibitory action of the ionophores on chloride *exchange was not* directly disproved

We used an ionophore gramicidin to increase the *cation* permeability Gramicidin is a linear polypeptide antibiotic of molecular *weight c 1880* isolated from Bacillus brevis (Sarges & Witkop 1965) Gramicidin *contains* many non-polar side chains and no ionozable groups as both the C *and N* terminals are blocked Gramicidin *forms channels* consisting of gramicidin dimers through thin lipid membranes The *channels include* monovalent cations and exclude anions like chloride and divalent cations (Myers & Haydon 1972) Reviews on gramicidin have been presented by Haydon & Hladky (1972) Hladky *(1974) and* Bamberg et al (1977)

We demonstrate that gramicidin turns the human red blood cell into a highly cation selective *membrane* with an unaltered chloride self-exchange which at neutral *pH is orders of* magnitude faster than the KCl diffusion The calculation of *permeability* coefficients from net salt transport was simplified because the experiment was designed as a special case of the Nernst Planck *diffusion regime* i e the single salt diffusion case The term of *the electrical driving* force is eliminated in the equation of the permeability coefficient in this *experimental* situation Some of the results have been cited previously (Cass & Dalmark 1974)

METHODS AND CALCULATIONS

Freshly drawn heparinized human red blood cells were washed thrice titrated to pH 6 8 (25°C) rewashed and resuspended to a hematocrit of 0.25 in a salt solution (150 mM KCl 27 mM sucrose) The buffy coat was removed

The temperature sensitivity of the gramicidin effect (cf Fig.) demanded that gramicidin—in all experiments—had to be added to the cell suspension at a high temperature in order to increase the cation permeability [illegible] temperature experiments were started one [illegible] was added to the cell suspension, (*b*) low tempe[illegible] experiments were carried out with cells incubated gramicidin at room temperature for half an hour before experiments were started Only insignificant [illegible] potassium exchange was observed after subsequent washes of the (b)-cells in gramicidin-free salt solution low temperature The gramicidin-treated cells were [illegible]ed to pH 7 (0°C) and resuspended in a salt solution (150 mM KCl 7 mM sucrose). The distribution [illegible] KCl between cell water and medium was [illegible] these conditions (Dalmark 1975*b*) Gramicidin was [illegible] as a methanolic solution of Gramicidin N.F "[illegible] (Lundbeck and Co. Copenhagen DK 2500) which [illegible] composed of gramicidin A 50% gramicidin B 7 gramicidin C 25%. Three types of experiments were carried out with gramicidin-treated cells and normal cells

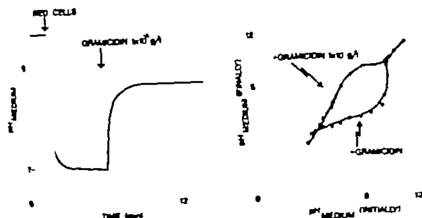
(1) The *low ionic strength experiments* were [illegible] injection of appr 700 mg packed cells into 30 ml [illegible]ly stirred medium containing 1 mM KCl, 300 mM [illegible] The medium pH was monitored by a pH-meter [illegible] ter Copenhagen, Denmark). A steady pH-value was [illegible] tained appr 1 min after an acid or base pulse.

(2) The *exchange experiments* were carried out as [illegible]scribed by Dalmark & Wieth (1972) by injection of [illegible] 200 mg packed and isotope labelled cells into 40 ml [illegible]ously stirred medium and, subsequently by serial [illegible]ing cell-free efflux medium by rapid filtration of the suspension The exchange kinetics of potassium [illegible] chloride was well described by a closed two-compar[illegible] model with constant volumes up to at least [illegible] isotopic equilibrium value The half-time of K [illegible] exchange was determined from a plot of ln (1−a/a_∞) [illegible] time where a or a_∞ are the specific activities at the [illegible] of sampling or at isotopic equilibrium. The slope [illegible] graph was equal to the rate coefficient k for [illegible] efflux under the conditions employed. The ch[illegible] equilibrium flux (influx = efflux) was calculated from [illegible] equation

$$M_{Cl}\ (\text{mmol}/3\times10^{13}\ \text{cells min}) = k \times (Cl)_c \times l \times F$$

where k is the rate coefficient of tracer efflux [illegible] $(Cl)_c$ is the cellular chloride concentration (mmol/[illegible] water) l is the cellular water content (kg water/[illegible] solids) and F has the dimension kg cell solids per 3 [illegible] cells (Dalmark 1975*a*) The 3×10^{13} cells have a [illegible] area of 4 890 m² (Ponder 1948) and contain 1 kg cells at an ionic strength of 0.15 (Funder & Wieth 1966)

(3) The *n[illegible]t salt transport experiments* were carried [illegible] with cells pretreated exactly as the cells of the exch[illegible] expts The expts were initiated by resuspending the [illegible] to a hematocrit of 0.25 in a salt solution (165 mM [illegible] *i.e there was a salt concentration difference of 15 mM* across the cell membrane at the beginning of the e[illegible] *and subsequently the cells* began to take up salt A[illegible] time of sampling the cells were separated from the [illegible]pending medium with an *amount of trapped extracel*[illegible] fluid of [illegible]% (w/w) by ultrafiltration The cellular [illegible] content was measured by drying packed cells to con[illegible] weight (105°C 4 h) The cellular content of chloride



1 The pH-shifts of low ionic strength medium following suspension of red cell in the medium and later on, after addition of gramicidin to the cell suspension (Fig 1). Acidification and alkalization of low ionic strength media following suspension of red cells in the media (hematocrit 0 01 v/v) as functions of the pH in the media prior to the suspension of the cells in absence (●) or in presence (O) of gramicidin (10^{-4} g/l) (Fig. 1*b*). The cells were washed in salt solution (150 mM KCl, 27 mM sucrose) and titrated to pH 6 6 (38°C) before the cells were suspended in the low ionic strength media (1 mM KCl, 302 mM sucrose) at 38°C The pH of the suspending medium was adjusted with KOH or HCl before the cells were suspended

determined either ( ) by coulometric titration (CMT chloride titrator Radiometer Copenhagen Denmark), or ( ) from the distribution of ^{36}Cl between cell water and plasma together with the chloride concentration in plasma. The potassium content in the cells was determined either ( ) by flame photometry (Radiometer Copenhagen, Denmark), or (*b*) from the potassium concentration in medium and the distribution of ^{42}K. The potassium and chloride concentration in medium was determined by flame photometry and coulometric titration. The equation of the permeability coefficient employed in net transport experiments is derived in Results.

RESULTS

The results will be presented in 4 sections. The first section describes the effect of gramicidin on ionic selectivity of the red cell membrane at low ionic strength. The second and third section contain experimental data on ( ) potassium and chloride self exchange and (*b*) net transport of potassium chloride and water at normal ionic strength. The last section contains an analysis of the net transport experiments and the estimate of the chloride permeability coefficient.

Effect of gramicidin on selectivity

A rapid acidification of the medium occurs when normal red cells are suspended in low ionic strength media at neutral pH (Davson 1939 Wilbrandt 1940)

It is generally believed that anions like chloride and hydroxyl ions rapidly reach thermodynamic equilibrium The distribution of chloride across the untreated membrane determines the pH of an unbuffered KCl solution because the cellular pH is constant owing to the high buffer capacity of hemoglobin Fig. 1 shows the acidification of a low ionic strength medium following suspension of red cells in the medium Subsequent addition of gramicidin to the cell suspension shifts the pH in the alkaline direction. Although the mechanism of the acidification is debated the alkalization of the medium following addition of gramicidin appears to be a transport of protons because proton permeability is greatly increased in gramicidin-treated thin lipid membranes (Myers & Haydon 1972) The potassium diffusion potential in gramicidin-treated red cells determines the distribution of protons across the cell membranes in the experimental situation employed.

Fig. 1*b* (filled circles) demonstrates the acidification of the medium as a function of the pH prior to normal cells were suspended in an unbuffered low ionic strength medium: the change in pH increased with the pH initially present in the medium. No change in pH was observed at an initial pH of 4.6. This pH value was predictable from the chloride distribution across the cell membranes the activity

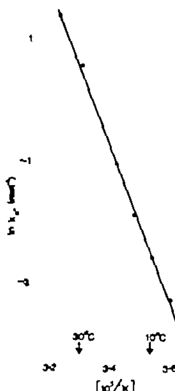


Fig 2 The Arrhenius plot showing the rate constant *k* (min) of proton flux across the red cell membrane after addition of gramicidin (10^{-4} g/l) to cells suspended in an unbuffered low *ionic strength medium* (hematocrit 0 01 v/v) as a function of the reciprocal absolute temperature (°K) The cells were washed in a salt solution (150 *mM* KCl 27 mM sucrose) and titrated to pH 6.8 (25°C) before the cells were suspended in the low ionic strength medium (*1 mM* KCl 302 *mM* sucrose neutral pH) The relationship between ln*k* (*Y*) vs 10^3/°K (*X*) was described by the equation *Y* = −12 7*X* + 42.3 *r* = −0 99 *The* Arrhenius activation energy of proton flux following *addition* of gramicidin was 105 kJ/mole (25 kcal/mole)

coefficients of chloride and the intracellular pH of 6 6 at 38°C The selectivity of the membranes disappeared at *pH* above nine A large increase in cation permeability has previously been demonstrated as an effect of a high *pH in the suspending* medium (Passow 1964 LaCelle & Rothstein *1966)*

Addition of gramicidin alters the cell membrane to a highly cation permeable membrane as demonstrated by the upper curve (open circles) in Fig 1*b* a pH shift was measured of a similar size but of an opposite sign as that observed with an anion selective membrane under identical conditions except for gramicidin The disappearance of selectivity above pH nine was also observed with gramicidin-treated cells

Fig 1*b* demonstrates that the potassium permeability is increased relative to chloride permeab in the pH range from five to nine in the presence of gramicidin The gramicidin molecule contains no ionizable groups as both the C and N terminals are blocked (Sarges & Witkop 1965) and gramic modified artificial lipid bilayers are highly cat selective even at low pH (Myers & Haydon 197

Formation of cation permeable channels is a complicated process since gramicidin is slightly soluble both in water and in lipids and furthermore the cation permeable unit is believed to be a dimer (reviewed e g Hladky 1974) Fig 2 demonstrates the high temperature sensitivity of the formation cation selective membranes following addition of gramicidin to the suspending medium Fig is an Arrhenius plot of the rate constant of the proton flux initiated by addition of gramicidin to a suspension of red cells in an unbuffered low ionic strength medium The rate constant was determined from the slope of a plot of ln ($\Delta H_t/\Delta H_\infty$) vs. time, where ΔH ΔH_∞ are the cumulative proton transport at the time *t* and at the new steady state No change in medium *pH* was observed at 0°C within app 1 h after addition of gramicidin. This temperature sensitivity demanded that the cells had to be treated with gramicidin at room temperature before the cells were employed in low temperature experiments (cf Methods)

Effect of gramicidin on potassium and chloride exchange

The potassium and chloride exchange across the human red cell membrane can be determined simultaneously at 0°C using a simple filtration den (Dalmark & Wieth 1972) The chloride exchange of normal cells has been studied extensively at the temperature (Dalmark & Wieth 1970 Gunn et 1973 Dalmark 1975*a*) Fig 3 shows the influence gramicidin on potassium and chloride exchange The half time of potassium exchange decrease drastically with gramicidin concentration (Fig in contrast to an unaltered half-time of chloride exchange (Fig 3*b*) The half-time of potassium exchange in normal cells in 35 days under conditions (Dalmark 1975*b*) but the half-time decreased to 1 s in the presence of 50 *µ*M gramicidin The slope of the line (Fig 3*a*) is smaller than the expected slope of two for a dimer channel However the relationship between the conductance of artificial bilayers and the gramicidin concentration also demonstrates a slope of less than

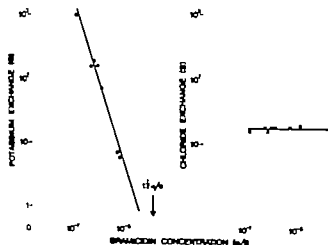


Fig. 3. The effect of gramicidin on potassium exchange (Fig. 3) and on chloride exchange (Fig. 3*b*) determined simultaneously at distribution ratio of potassium chloride between cell water and medium of unity (pH 7.., 0°C). The composition of the medium (mM): 150 KCl, 27 sucrose. The lines were drawn by eye ( ) with slope of 1.3 (Fig. 3), and (*b*) with slope of zero (Fig. 3*b*). Assuming that the membrane potential difference is equal to zero, reasonable assumption in view of the salt distribution ratio of unity the "tracer permeability coefficient" (*P*) is given by the equation: *P* (cm/s)=(3 10⁻⁴)/t½ where ½ is the half-time of tracer exchange (seconds). The gramicidin was added to the cells at room temperature (cf. Methods).

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Similar exchange studies were performed at 25°C at pH 6.8 because the electrical net charge on the intracellular buffers is zero at this pH and temperature. Chloride exchange in normal cells has a half time of appr. 0.2 s (Tosteson 1959, Brahm 1977). We demonstrated with our manual filtration technique that chloride tracer was at isotopic equilibrium within 2 s regardless the gramicidin concentration although potassium exchange increased drastically at 25°C as at 0°C (Fig. 3*a*).

Effect of gramicidin on net potassium chloride transport

The cells of the exchange experiments were suspended in 150 mM KCl plus 27 mM sucrose at pH 6.8 (25°C) or at pH 7.2 (0°C). The potassium chloride distribution ratio between the concentrations in cell water and medium was unity under these conditions (Dalmark 1975*b*) and no net transport of salt took place.

The cells of the following net salt transport expts were pretreated with gramicidin as described in Methods and resuspended in 165 mM KCl where upon the cells began to take up potassium chloride and water in the presence of a concentration differ

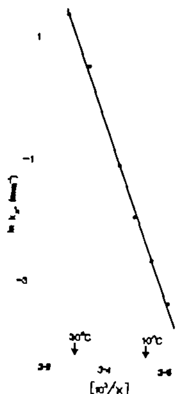


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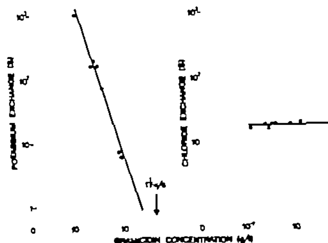


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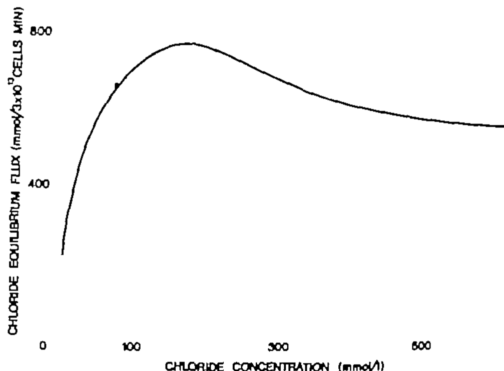


Fig 4 The dependence of chloride equilibrium flux on chloride concentration (pH 7.2, 0°C) in the presence of gramicidin (10^{-4} g/l) compared with chloride exchange in normal cells (−) taken from Dalmark (1975*a*). The cell concentration of salt was altered by the nystatin technique (Cass & Dalmark 1973) The electrolyte solutions contained (mM) 50–500 KCl 27 sucrose The distribution ratio of KCl between cell water and medium was unity The cells were treated with gramicidin at room temperature (cf Methods)

ence of 15 mM KCl across the cell membrane Fig 5 shows simultaneous values of the cellular potassium and chloride content in a net transport experiment One observes that the cells took up potassium chloride Fig 6*a* shows the cellular water content as a function of time after addition of gramicidin in a net transport experiment Fig 6*b* shows the cellular water content as a function of the sum of cellular potassium and chloride content. The transported fluid was slightly hyperosmotic as 2 64 g water was transported concomitantly with one mmol ion against the expected 3 03 g water/mmol ion for a solution of 165 mM KCl The imperfect osmotic behaviour of red cells has been described under various experimental conditions (Ponder 1948 Dalmark 1975*b*) A net uptake of potassium chloride accompanied by a hyperosmotic water transport was also demonstrated at 0°C (pH 7 ?)

Theoretical analysis and the estimate of chloride permeability

In this section we develop expressions for the net transport of a uni-univalent salt across a membrane having a highly cation selective region and a highly anion selective region We imagine these two re-

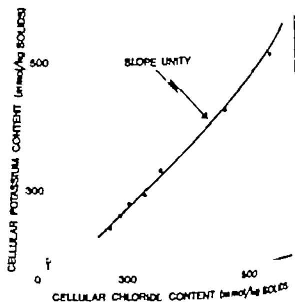


Fig 5 The cellular content of potassium vs the cell content of chloride in cells from one net transport experiment (25°C pH 6 8) The cells were prior to the experiment equilibrated with a salt solution (150 mM KCl mM sucrose) and resuspended in another salt solution mM KCl) The experiment was started by addition of gramicidin (10^{-4} g/l) and the cells took up KCl in the presence of a KCl concentration difference across the cell membrane At the appropriate times the cells were analyzed for potassium chloride and water

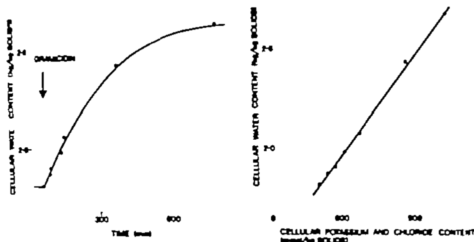


Fig. 6 The cellular water content as function of time after addition of gramicidin (Fig. 6a) and as function of the sum the cellular content of potassium and chloride (Fig. 6b) in net transport experiment (25°C, pH 6.8). The experimental conditions are described in legend to Fig. 5 The following equation was obtained by regression analysis of the cellular water content, Y vs the sum of the cellular content of potassium and chloride X Y (kg water/kg cell solids)=0.00264X (mmol ion, potassium or chloride/kg cell solids)+0.34963 =0.998.

ions to be physically separated in the membrane although the expressions we obtain will be similar to those derived from the Nernst Planck flux equations assuming that the ions pass through a single and homogeneous regime. The reason for assuming that the regions in our red cell membrane are physically separated is that we have created the cation selective region artificially using gramicidin. We want to determine the permeability of the naturally occurring chloride selective region—or in any case to demonstrate which fraction the chloride transport by diffusion constitutes of the total chloride transport in gramicidin-treated human red cells

We want to calculate net potassium chloride flux V_{KCl}, through a mosaic membrane containing gramicidin channels in the presence of a potassium chloride concentration difference Δ(KCl) The only permeating species in cells and medium is potassium chloride and the potassium and chloride concentrations are under the conditions employed described by

$$(K)_c \quad (Cl)_c + (K)_m = (Cl)_m \qquad (1)$$

This condition is only fulfilled at a given temperature at one single pH value i.e. where the electical net charge on hemoglobin is zero

We assume that (1) the gramicidin-induced permeability is much greater than the endogeneous potassium permeability in the membrane, which we neglect, and (2) the chloride permeability is unaffected by gramicidin.

Fig. 7 shows the suggested equivalent circuit of the membrane The batteries, E or E_{Cl} represent

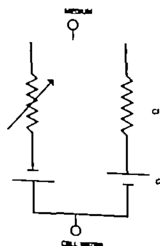


Fig 7 The equivalent circuit representing the cell membrane of gramicidin-treated human red blood cells. The potassium and chloride concentration cells, E_K and E_{Cl}, arranged in parallel are the main sources of membrane current while R and R_{Cl} represent the resistance of the regions through with these ions pass The dependence of R on gramicidin concentration is depicted by the arrow

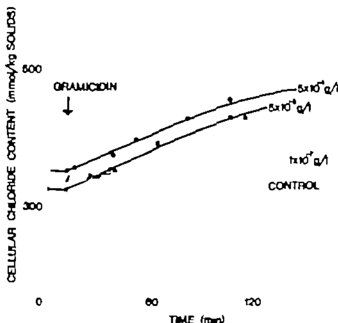


Fig 8 The cellular content of chloride as a function of time after addition of various doses of gramicidin in 4 net transport experiments (25°C pH 6.8) The experimental conditions are described in legend to Fig 5 except for the gramicidin doses The drawn curves are parallel at the two highest gramicidin doses indicating that chloride at high gramicidin concentration is the rate-limiting ion of the KCl flux

the potassium and chloride concentration cells while R_K or R_{Cl} represent the resistances of the regions through which the ions pass The R_K varies with the gramicidin content in the membrane

The current through either limb of the circuit may be written

$$I_K = g \; (E_K - E_m) \qquad (2a)$$
$$I_{Cl} = g_{Cl}(E \;_1 - E_m)$$

where I_K or I_{Cl} are the potassium or chloride current densities in the inward direction g or $g\;_1$ are the corresponding conductances per unit area and E_m is the electrical membrane potential difference with the outside solution as a reference The potassium and chloride batteries are defined by (cf (1))

$$E_K = (RT/F)\ln((K)_m/(K)_i) = (RT/F)\ln((KCl)_m/(KCl)_i) \qquad (3a)$$

$$E_{Cl} = (RT/F)\ln((Cl)_m/(Cl)_i) = (RT/F)\ln((KCl)_m/(KCl)_i) \qquad (3b)$$

where R is the universal gas constant T is the absolute temperature the valence and F is Fara-day s constant

Hence

$$E_K = -E_{Cl}$$

At steady state of transport we have the zero cur-rent condition

$$I_K + I_{Cl} = g_K(E_K - E_m) + g_{Cl}(E_{Cl} - E_m) \;\; 0 \qquad (5)$$

and the membrane potential difference is given b

$$E_m = (g_K - g_{Cl})E_K/(g_K + g_{Cl})$$

At steady state of transport the fluxes are give by

$$M_{KCl} = M_K = M \;_1 = I_K/F = I \;/F$$

where M_{KCl} M_K and M_{Cl} are the fluxes of neu salt of potassium and of chloride per unit ar From (2a) (3a) (5b) and (6a) we obtain the flux neutral salt

$$M_{KCl} = (RT/F^2)(2g_K g_{Cl}/g_K + g \;_1)) \ln((KCl)_m/(KCl)_i)$$

We expand the natural logarithm after defining th potassium chloride concentration differenc $\Delta(KCl) = (KCl)_m - (KCl)_i$ noting that $\Delta(KCl)/(KCl$ is much less than unity and we obtain

$$M_K \;_1 = 2\Delta(KCl)/((F^2(KCl)_i/RTg_K) + (F^2(KCl)_i/RTg \;_1))$$

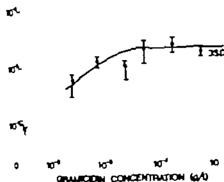
Defining the potassium permeability coefficient P and the chloride permeability coefficient P b

$P_K = RTg_K/F^2(KCl)_i$ and

$P_{Cl} = RTg \;/F^2(KCl)_i$ we obtain from (6c)

$$M_{KCl} = \; P \; P \;\; \Delta(KCl)/(P \; + P \;_1) = P_{KCl} \; \Delta(KCl)_i$$

which is Fick s equation of single salt flux whe $P \;_{Cl}$ is an operationally defined apparent perme bility coefficient of KCl equal to $P \; P_{Cl}/(P \; + P \;)$



9 The permeability coefficient of potassium chlo-
, P_{KCl}, as a function of gramicidin concentration in
suspending medium (pH 6.8 25°C). The P_{KCl} is an
nominally defined apparent permeability coefficient of
derived in Results for the net transport experiment,
the single salt diffusion case.

The salt flux will increase with gramicidin—as
potassium permeability increases—to a finite
lue which is determined by the chloride permea-
lity (cf. (6 d)) If experimentally we have that
is much less than the measured P then P_{Cl} will
termine the value of P_{KCl} and chloride will be the
te-limiting ion of salt flux, since

$$_{KCl}=(2P_{Cl})/(1+(P_{Cl}/P_{K}))\approx 2P_{Cl} \quad (7)$$

and we have

$$J_{KCl}=P_{KCl}\,\Delta(KCl)\approx 2P_{Cl}\,\Delta(KCl) \quad (8)$$

Fig. 8 shows that net potassium chloride flux
creased with gramicidin concentration to a finite
vel in contrast to the continuously increasing
potassium flux (cf Fig 1 and Fig. 3*a*) So the
experiments performed with a high gramicidin con-
centration fulfilled the requirements of eq 8

Fig. 9 shows the calculated P_{KCl} as a function of
gramicidin concentration. One observes that P_{KCl}
increased with gramicidin concentration until a
plateau where P_{KCl} was gramicidin-insensitive. The
gramicidin-insensitive P_{KCl} (pH 6.8, 25°C) was
4.2×10^{-8} cm/s S.D $2.9\times10^{-}$ = 16, indicating a
P = $\times10^{-8}$ cm/s.

The gramicidin-insensitive P_{KCl} at 0°C (pH 7.2)
was 1.3×10^{-8} cm/s S.D 0.7×10^{-8} = 17 indicat-
ing a P_{Cl} of 6.5×10^{-9} cm/s. The Q_{10} was app three
indicating an Arrhenius activation energy of app 40 kJ/mole (10 kcal/mole)

DISCUSSION

The paper demonstrates a marked discrepancy in *gramicidin-treated human red blood cells* between the chloride permeability coefficient calculated from (1) chloride exchange and (2) net salt transport in the presence of a concentration gradient of salt. The calculation of the chloride permeability coefficient in the net salt transport study was simplified since the experiment was designed as a special case of the Nernst Planck diffusion regime i e the single salt diffusion case It is unnecessary in this situation to know the actual membrane potential difference as the term of the electrical driving force is eliminated in the equation of the permeability coefficient The electrical membrane potential difference and therefore the electrical driving force was supposed to be zero in the exchange study because the distribution ratio of potassium chloride between cell water and medium was unity

The chloride permeability coefficient calculated from tracer experiments at anion equilibrium is 1.9×10^{-6} cm/s (0°C, pH 7.2) regardless the gramicidin concentration (Fig. 3*b*) This value is 290 times the chloride permeability coefficient calculated from net salt transport experiments at high gramicidin concentrations (0°C, pH 7.2) These observations demonstrate that the slow chloride net transport compared to chloride exchange is not a gramicidin-induced decrease in the transport rate of the chloride transport apparatus which only for the reason of electroneutrality has to work like a chloride exchange mechanism in normal cells

Are these results on chloride transport in gramicidin-treated cells valid in normal cells? Qualitatively it appears that chloride transport by diffusion also in normal cells only makes up a small fraction of the total chloride transport since (1) the characteristics of chloride exchange is unaffected by gramicidin (Fig. 4) and (2) cells containing gramicidin channels have a chloride permeability coefficient much less than expected from chloride exchange The magnitude of this fraction is difficult to assess since the exact value depends on the selectivity of the gramicidin channels. Myers & Haydon (1972) noted that the transference number of chloride was 0.2 in gramicidin-treated artifical bilayers separated by calcium chloride These

membranes are very low conductance membranes as calcium is almost totally excluded from the gramicidin channels Consequently it is difficult to exclude the possibility that the chloride diffusion in red cells increases with gramicidin concentration However (1) the chloride permeability coefficient is independent of gramicidin concentration at high gramicidin concentrations (Fig 8) and (2) the value of the chloride permeability coefficient presented here agrees roughly with values obtained by investigators using valinomycin as cation transport inducing substance (Hunter 1977 Knauf et al 1977)

Gramicidin has previously been used to investigate the chloride permeability in human red cells under various conditions Qualitatively Scarpa et al (1970) pointed out that the electrogenic chloride transport was much smaller than expected from data on chloride exchange given in the literature Wieth Dalmark Gunn & Tosteson (1973) determined a chloride permeability coefficient of $1\ 2\times10^{-7}$ cm/s (25°C pH 6 8) from potassium chloride net flux into a low ionic strength medium in the presence of gramicidin and phloretin a potent inhibitor of chloride exchange This value is 6 times greater than the value presented here The difference might be an effect of either the low ionic strength which is known to increase cation permeability (LaCelle & Rothstein 1966) or the presence of phloretin a aglycone of phlorizin which is known to increase chloride permeability (Kaplan & Passow 1974)

The chloride permeability coefficient in unmodified red cells can be determined by measuring the electrical conductance of the cell membrane as the measuring technique is insensitive to a large electroneutral chloride transport by exchange However impalement of the tiny cells with glass electrodes invariably causes a leak around the electrode by which a rapid discharging of the membrane potential takes place This difficulty is especially pronounced with the small human red cells (Lassen & Sten-Knudsen 1968 Jay & Burton 1969) The problem with the rapid discharging has probably been circumvented in the measurements of the membrane resistance in the large red blood cell of the Amphiuma In that case resistances as high as 10 ohmcm² (18°C) has been reported (Hoffman & Lassen 1971 Lassen 1972 Lassen et al 1974 1978) These values should be compared with a "chloride resistance" of 4 ohmcm ² calculated from chloride exchange in the same type of cells

The chloride diffusion has a low temperature sensitivity with an activation energy of app. 42 kJ/mole (10 kcal/mole) in contrast to chloride exchange with an activation energy of 84–126 kJ/mole (20- kcal/mole) (Dalmark & Wieth 1972, Brahm 1977). The chloride exchange is orders of magnitude faster than chloride diffusion with a highly temperature-dependent factor 2.9×10⁶ (0°C) 0.7×10⁶ (25°C) and ?×10⁴ (body temperature).

In conclusion the original suggestion by [illegible] (1922) is still valid the red cell membrane is selectively anion permeable in the sence that anion permeability exceeds cation permeability The [illegible] membrane is anion selective with a factor of $1\ 3\times10^{2}$ (0°C pH 7 ?) as the passive potassium permeability coefficient under identical conditions (temperature pH cell and medium composition) is 5×10^{-11} cm/s (Dalmark 1975b). The membrane selectivity lasts from 0°C to body temperature [illegible] both chloride and potassium diffusion has a low temperature sensitivity The physiologic [illegible] change i e the Hamburger shift, is a tightly coupled transport process where anion influx and efflux are linked together by interaction between the anions and groups attached to the cell membrane This anion transport by exchange is orders of magnitude faster than chloride transport by independent migration

I am glad to express my indebtedness to Mrs [illegible] Olsen for her valued technical assistance. This work [illegible] supported by a grant from The Danish Heart Foundation Copenhagen Denmark

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letabolic properties of nerve endings olated from rat brain

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JANSSON S.-E HÄRKÖNEN M. H. & HELVE, H. Metabolic properties of nerve endings isolated from rat brain. Acta Physiol Scand 1979 107 205–212. Received 23 Jan. 1979 ISSN 0001-6772. Department of Clinical Chemistry University of Helsinki, Finland.

Energy metabolism was studied in nerve endings isolated from 3-week-old rat brain. Concentrations of glycogen glucose ATP phosphocreatine and lactate were lower in synaptosomes than in the intact brain. The consumption of these endogenous substrates, the ability to generate high-energy phosphate and the production of ammonia were determined in aerobic and anaerobic conditions. Unlike nerve tissue in general, synaptosomes preferentially utilized endogenous ATP and phosphocreatine stores which, on incubation in the absence of exogenous substrates, were emptied long before glycogen stores were exhausted. The optimal medium for respiratory studies was found to ha e electrolyte concentrations equal to the extracellular fluid. The synaptosomes had an endogenous respiration rate of 6.3 nmol O_2 mg prot. min, measured with an oxygen electrode, and it probably reflects consumption of their glycogen stores. Glucose usually had no effect on the respiration rate of synaptosomes, but sometimes increased it slightly However after incubation in the presence of arsenate synaptosomes showed an increase in respiration when glucose was added ADP when added with glucose, also stimulated respiration. Pyruvate and succinate always increased respiration, succinate usually having the stronger effect. The present results show that isolated nerve endings are metabolically intact, which justifies their use in research on neurotransmission. In addition, opposite to the present consensus, synaptic transmission does not seem primarily to depend on the availability of glucose but rather on local stores of high-energy phosphate compounds.

Key words Synaptosomes, respiration, energy metabolism, synaptic function

Present knowledge of the metabolism of nerve endings is limited and based mainly on the results of studies on the oxygen consumption of isolated nerve endings (synaptosomes) Such studies have shown that synaptosomes respire using glucose, pyruvate (Bradford 1969) glutamate and succinate (Verity 1972) as substrates, and that ATP and phosphocreatine can be synthesized in the presence of glucose (Bradford 1969). Little attention has been paid to the substrates that provide energy for synaptosomes, despite the observations that synaptic transmission is the aspect of nerve function most vulnerable to shortage of glucose (Larrabee & Klingman 196.., Härkonen et al. 1969).

The present study concerned the oxygen consumption, high-energy phosphate production and rates of energy usage by synaptosomes in various conditions. The results indicate that synaptosomes are metabolically intact and that local energy metabolism plays an important role in synaptic transmission.

MATERIAL AND METHODS

Preparation of synaptosomes Three-week-old Sprague Dawley rats of both sexes were decapitated and the brain, except for the cerebellum, was dissected out and homogenized in 0.32 mol/l ice-cold, oxygenated sucrose in glass homogenizer with Plexiglass pestle (clearance 250 µm) at 850 rpm for 2 min. In some experiments, the cerebral cortices were dissected out and used instead of whole-brain homogenates. The nuclear and crude mitochondrial fractions, and the subfractions containing myelin, synaptosomes and mitochondria ere isolated as described by Gray & Whittaker (1962). After one wash the synaptosomes were resuspended in 0.32 mol/l sucrose. The protein of the synaptosome suspension was determined according to Lowry et al. (1951). The purity of the fractions were checked by electron microscopy

Incubation techniques. When oxygen consumption was determined, sample of the synaptosome suspension in sucrose was diluted with 1 ml of incubation medium and incubations were carried out in thermostated Plexiglass vessel at 30°C. In other expts., the diluted synaptosome preparations were incubated at 37°C in stoppered 50-ml polypropylene centrifuge tubes (gentle shaking), into which gases (95% O_2+5% CO_2 or N_2) were bubbled through venipuncture needles. Samples were withdrawn

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ble 2. *The concentrations of various substrates of energy metabolism in isolated nerve endings and in cerebral cortex of the rat (mmol/kg prot.)*

- ues referring to synaptosomes and cerebral cortex are means of at least two determinations or means ± S.E. of 4 terminations

		Cerebral cortex	
tabolite	Synaptosomes	Immediately frozen[a]	Delayed frozen[b]
ycogen	4 20±0 51	28.8±0 66	18.0±0.22
ucose	0.30	4 05±0.68	1 47±0.36
P	2 81±0.34	17.8±0.06	5.86±0.82
reatine	2.29±0 43	11 4±1.98	2.19±0.65
ctate	5.22±1 41	24.4±0.83	34 0±2.64
P	94±0.36		
P	4.93±0.95		
MP[c]	0 031	0.020	

Head frozen in liquid nitrogen immediately after decapitation.
Skull opened and brain removed before freezing in liquid nitrogen.
Determined according to Raij & Harkönen 1976.

mol/l. At 0.5–4 mmol/l Ca^{2+} respiration was ghtly enhanced (8–10%) but at higher concentra- ons (8 mmol/l) clearly depressed. Mg^{2+} also slight- enhanced respiration at concentrations of 0.5–5 0 mol/l. Neither ion had any stimulatory effect on spiration in the presence of 10 mmol/l pyruvate or cinate. No change in oxygen uptake occurred in ucose pyruvate or succinate medium when the O^{3-} (0.5–10 mmol/l) or K (1 20 mmol/l) concen- ation was increased stepwise. The negative results ith high K concentrations could in part be due to e fact that the ynaptosomes might ha e been epolarized during storage in the cold sucrose solu- ion. The short incubation time in the electrolyte edium was not sufficient to repolarize the synap- osomal membrane In tests with various buffers midazole-HCl, Tris-HCl, glycylglycine, bicarbo- nate) none seemed to be superior to the others.

The isolated synaptosomes contained large mounts of endogenous substrates and respired at n appreciable rate without addition of substrates (6.3±1.0 nmol O_2/mg prot./min, mean ±S.E. of 8 synaptosomal preparations, range 4.02 12.4 nmol O_2/mg prot./min). Oxygen consumption was linear during the first 3–5 min (the values given in Table 1 were measured within this time) but then often tended to decrease. Thus, when synapto- somes were incubated in oxygenated substrate-free medium at 30°C, respiration fell to about 50% in 30 min and to 20% in 60 min. Endogenous respiration as not significantly lowered when synaptosomes were stored at 0°C for as long as 6 h, although sometimes the response to added substrates seemed to be weaker than that of freshly prepared synapto- somes.

Addition of 10 mmol/l glucose to the incubation medium did not have a constant effect (Table 1). Mostly respiration was unaffected, but in some ex periments small increase was seen Even with synaptosomes which had been aged by incubation at 30°C for 30–60 min in the absence of substrates, addition of glucose did not raise oxygen consump- tion significantly However when synaptosomes were first incubated in 40 mmol/l arsenate for 1 h, which by itself did not stimulate respiration addi- tion of glucose induced a -fold increase in oxygen consumption Addition of 5 mmol/l ADP to a glu- cose medium produced a 70–100% increase in re spiration (Table 1). The response was not linear and the highest values were measured during the first few minutes.

Pyruvate and succinate at a concentration of 10 mmol/l stimulated oxygen uptake, raising it to twice the level without any added substrate (Table 1). Respiration with pyruvate was not linear and after a few minutes only 40% of the effect remained.

FCCP (carbonyl cyanide p-trifluoromethoxy phenylhydrazone) and oligomycin were used to study the mechanisms controlling the respiration of synaptosomes. In a glucose medium, 1 mmol/l of FCCP had a slight stimulatory effect (34% mean increase) on the rate of oxygen consumption (Table 1) The effect of oligomycin was also small but in 4 expts. the addition of 1 μg/ml oligomycin after

Table 1 *Effect of substrates or inhibitors on endogenous respiration of synaptosomes*
Endogenous respiration was 6.3±1 0 nmol O_2/mg prot./min (mean ± S.E. of 8 expts.). All expts. were carried at 30°C The increase (+) or decrease (−) in oxygen uptake induced by an agent was calculated in comparison to the ... respiration in the particular experiments Values are means ± S.E. of 4–14 expts. indicated as the number of different batches of synaptosomes/the total number of tests

Agent added	Effect of oxygen uptake nmol O_2 mg protein^{-1} min	Change (%)	No. of expts.
Glucose 10 mmol/l	+0 33±0.30	+ 5	8/14
Pyruvate, 10 mmol/l[a]	+5 90±0.45	+89±6	3/10
Succinate 10 mmol/l	+6 4±0 48	+83±4	4/12
ADP 1 mmol/l[b]	+1 46±0 16	+ 4±3	./7
ADP 5 mmol/l	+5 08±1.35	+52±2	4/4
FCCP 10−[c] mmol/l	+2.54±0..8	+34±6	./7
Oligomycin 1 μg/ml[d]	−0 10	− 7	4/4

[a] During the first linear minute.
[b] The effect of ADP was studied in the presence of 10 mmol/l glucose
[c] FCCP was added after glucose or glucose plus 1 or 5 mmol/l ADP
[d] The effect of oligomycin was studied in the presence of 10 mmol/l glucose.

with a long needle without interruption of incubation and fixed immediately with an equal volume of 0 6 mol/l ice-cold perchloric acid containing 2 mmol/l EDTA. The mixture was centrifuged in the cold at 3 500×g for 5 min and a portion of the supernatant was neutralized (pH 6.5) with .5 mol/l $KHCO_3$.

Determination of oxygen consumption The oxygen consumption of synaptosome suspensions was determined with a Clark-type oxygen electrode (Clark et al 1953)

Determination of metabolites Glycogen ATP ADP AMP and lactate in the neutralized perchloric acid extract were measured by fluorometric enzymatic methods with a Farrand A 3 fluorometer (Lowry et al 1964 Passonneau et al 1967) As sucrose is partially hydrolysed in perchloric acid glucose was determined in a $Ba(OH)_2$–ZnSO extract of synaptosomes (Härkonen et al. 1969) Ammonia production was determined by a fluorometric enzymatic method (Folbergrova et al 1969)

Semi-quantitative evaluation of the vesicle content of incubated synaptosomes For electron microscope studies a sample of the synaptosome suspension was diluted with ice-cold 2.5% glutaraldehyde solution in 0 1 mol/l phosphate buffer pH 7 4 After fixation the synaptosomes were centrifuged and the pellet processed according to a conventional EM routine Photographic negatives of representative areas were taken at 10 500×magnification and after framing and coding projected onto white lined background The entire coded negative was independently inspected by two of the authors and every recognizable synaptosome was evaluated for its vesicle content by expressing the area filled with vesicles as a percentage of the whole area of the synaptosomes (except for the intrasynaptosomal mitochondria). Thus 100% denotes synaptosomes packed with vesicles whereas 0% denotes particles identified as synaptosome ghosts or as synaptosomes without intact vesicles. Every negative contained about 20 synaptosomes and each author evaluated about 100 synaptosomes in various states for each of the different incubation conditions. The validity of this subjective method of quantification was confirmed by planimetry and actual counting of vesicles.

Reagents The water used had been deionized and passed through charcoal and Millipore filters. Substrates and pyridine nucleotides were obtained from the Sigma Chemical Company (St. Louis Mo., USA) or from Boehringer and Sohne (Mannheim West Germany), lactate dehydrogenase was purchased from Worthington (Freehold N.J USA). Other chemicals used were commercially available products of analytical grade.

RESULTS

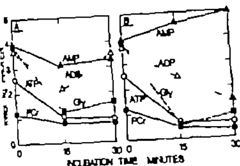
Purity of the synaptosomal fraction

The purity of every synaptosomal fraction prepared was checked by electron microscopy The nerve endings were mainly well-preserved but the fractions also contained some unidentified membrane profiles either empty nerve endings or fragments of glial origin. Mitochondrial contamination was less than 10% of all particles and consisted almost solely of small apparently synaptosomal mitochondria.

Oxygen consumption

Respiration was studied routinely in a medium consisting of NaCl 136 (values in mmol per liter), KCl 5 6 $CaCl_2$ 2.2 NaH_2PO 1 2 $MgCl_2$ 1.3 and imidazole buffer pH 7 4 30

The effects of Ca^{2+} Mg^{2+} K and PO ions on synaptosomal consumption of oxygen in the presence of 10 mmol/l glucose were tested by increasing the concentration of each ion from zero to 10−



. 4 Metabolism of high-energy phosphate compounds synaptosomes in an oxygenated electrolyte medium at The medium was supplemented with 10 mmol/l glucose (A) or with 10 mmol/l glucose+5 mmol/l ADP (B) ADP content of the blank incubation solution was tracted from the ADP values). Results of typical eriment

cult to prepare, glucose was not measured dur incubation studies. During the first 5 min glyco- dropped to 65% of the initial level and after 15 only about half of the original concentration s left (Fig. 1). The rate of consumption then wed down, but at 3 h practically all glycogen had en used up. Lactate increased during the first 5 n and then decreased at a constant rate and at 3 h ly trace amounts were detected. ATP and phos- ocreatine fell at comparable rates after 5 min e values were only about half the initial level d after 1 h less than 10% was left.

anges in metabolit during ubation in nitrogen

hen synaptosomes (3 separate preparations) were cubated in a nitrogen-saturated medium in the sence of added energy substrates, glycogen fell to lf the initial concentration in 10 min then de eased more slowly (Fig. 2) After an initial lag of 1 n the lactate concentration rose and the increase as roughly proportional to the decrease in ycogen. ATP and phosphocreatine fell rapidly ring the first min reaching 30% and 15% of the spective initial levels After 10–15 min, there was ss than 10% of these substrates left.

An approximate metabolic rate can be calculated r the synaptosomes from the rate of use of high- ergy phosphate (~P) seen as changes in ATP hosphocreatine, glucose and glycogen (see e.g. Erkonen et al. 1969) During the first min of anoxia P was used at a rate of 10 mmol/kg prot./min. If the P O ratio is taken as 3 this rate of use of ~P is equivalent to an oxygen consumption of 3.3 nmol/mg prot./min. This is somewhat lower than the value measured with an oxygen electrode.

Generation of ATP and phosphocreatine

Synaptosomes were incubated with constant oxygenation in a medium containing 10 mmol/l glucose alone or 10 mmol/l glucose and 5 mmol/l ADP in order to study their ability to synthesize high-energy phosphate. Neither alone nor with ADP could glucose maintain the initial level of these high-energy phosphate compounds (Fig. 3). However in the presence of glucose the decrease was smaller than during endogenous respiration, e.g. ATP decreased by 1.3 mmol compared with 2.. mmol and phosphocreatine by 0.3 mmol compared with 1 7 mmol per kg protein during the first 15 min (values are means of 2 separate expts.) Furthermore after this initial fall, the level of both compounds remained the same (ATP 1 0 and phosphocreatine 0.8 mmol/kg prot.) for 1 h while during endogenous respiration, high-energy phosphates were practically exhausted.

The presence of 10 mmol/l glucose could not prevent the concentrations of ADP and AMP from decreasing during incubation, especially during the first 15 min (Fig. 4A). In Fig 4B (incubation medium supplemented with 10 mmol glucose +5 mmol/l ADP) the linear decrease of ADP and the linear increase of AMP as a function of time, suggest the presence of enzymatic ADPase activity. Generation of high-energy phosphate was also studied in a Ca-free medium containing 20 mmol/l

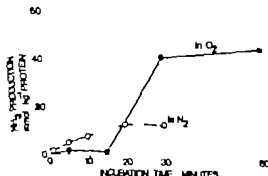


Fig 5 Ammonia production in isolated nerve endings in an electrolyte solution without added energy substrates in the presence or absence of oxygen at 37°C. Results of typical experiment.

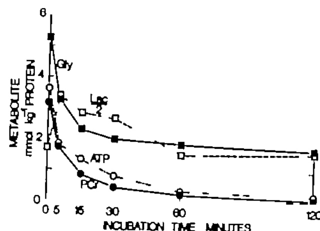


Fig 1 Utilization of energy metabolites in isolated nerve endings. Synaptosomes were incubated at 37°C in oxygenated substrate-free electrolyte solution buffered with imidazole—HCl pH 7 4 At the times indicated samples were withdrawn from the suspension Results of a typical experiment Gly=glycogen Lac=lactate and PCr=phosphocreatine

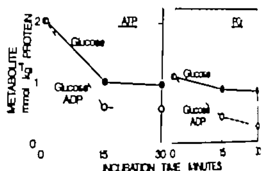


Fig 3 Generation of ATP and phosphocreatine in synaptosomes in an oxygenated electrolyte medium at 37°C The medium was supplemented with 10 mmol/l glucose or with 10 mmol/l glucose+5 mmol/l ADP Results of a typical experiment

glucose inhibited the respiration by 7% Likewise when oligomycin was added after 40 mmol/l arsenate 18% inhibition was obtained

Metabolite levels

Table 2 shows the concentration of energy metabolites measured in perchloric acid extracts of freshly prepared synaptosomes The metabolite concentrations were considerably lower in the synaptosomes than in cerebral cortex which had been frozen immediately The decrease was very rapid during the initial 15–20 s following decapitation while the brain was being dissected out and thus before it was chilled This became evident when the metabolites were measured in extracts of cerebral cortices obtained either after freezing the head of the rat in liquid nitrogen immediately after decapitation or after dissection in the same way as for preparation of synaptosomes followed by freezing (Table 2). ATP and phosphocreatine concentrations were only a little lower in the synaptosomes than in the "delayed frozen" cerebral cortex and corresponded well to levels previously reported (Barbers & McIlwain 1976 Hawthorne & Pickard 1977 Nelson Krause & Howard 1978) Thus during the actual isolation procedure the decrease was relatively slow This was apparently due to the fact that the synaptosomes had adequate supplies of oxygen and nutrients and metabolism was slowed down by chilling The differences in glucose and lactate levels between the synaptosomes and the "delayed frozen" cerebral cortex is probably due to diffusion of these compounds into the surrounding medium

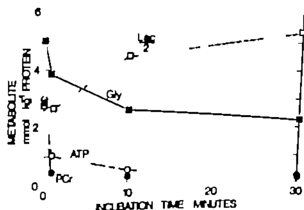


Fig 2 Effect of anoxia on the utilization of energy metabolites in isolated nerve endings. A suspension of synaptosomes was incubated under nitrogen in the absence of added substrates at 37°C, and samples were taken at the times indicated. Results of a typical experiment

Changes in metabolites during incubation in oxygen

The concentrations of glycogen ATP phosphocreatine and lactate were followed during incubation in oxygen for 2–3 h in a substrate-free medium in 3 different synaptosomal preparations (Fig 1) When synaptosomes are isolated in a sucrose gradient measurement of glucose is difficult because perchloric acid produces glucose via hydrolysis of sucrose For measurements in freshly prepared synaptosomes we therefore used the Ba(OH)₂–ZnSO precipitation method and found glucose to be approximately 0.3 mmol/kg prot. (Table 2) As the concentration was so low and extracts are

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The insensitiveness of our synaptosome preparation to glucose is probably due to the short initial servation time during which they were still respiring on endogenous glycogen stores. The failure to rease respiration in aged synaptosomes which d consumed most of their glycogen stores, may ve been due to tissue catabolism and damage to al enzymatic structures during the aging process. is explanation is supported by the observation at during the first 30 min of incubation, ammonia oduction was considerable.

The insensitiveness of our synaptosome preparation to uncouplers apparently reflects the fact that the complex metabolic system which the synaptosome represents, respiration is controlled at a ep hich is not affected by uncouplers.

The stimulating effect of ADP on glucose respiration leads us to assume that ADP despite its positive charge, does to some extent penetrate the synaptosomal membrane. Taking the ratio of the respiration rate in the presence of ADP to that in its absence as an indication of the respiratory control of the system (Chance & Williams 1956) a ratio of 7 is obtained with 5 mmol/l ADP which would suggest fair respiratory control. When intrasynaptosomal mitochondria have been isolated in Ficoll-sucrose media they have also shown good metabolic activity and respiratory control (Lai & Clark 1976)

Generation of high-energy phosphates

Bradford (1969) reported that, with glucose as substrate, synaptosomes synthesized both ATP and phosphocreatine Closer scrutiny of his data, however shows that actually he measured the levels of ATP and phosphocreatine in synaptosomes after incubation for 1 h in various media in the presence of metabolic poisons In our generation experiments we followed the concentration of both compounds during the course of incubation The rapid initial decrease in ATP concentration could be due to the membrane Na K -ATPase (Abdel-Latif & Abood 1964) activated by temperature and Na and K in the incubation medium. The simultaneous decrease in phosphocreatine could thus be due to creatine kinase activity tending to synthesize ATP from ADP and phosphocreatine. After the initial fall there was hardly any change in ATP or phosphocreatine concentration during the next 2–3 h Our interpretation of these results is that NA K – ATPase activity creatine kinase activity and oxidative phosphorylation supported by glucose (or glycogen), came to equilibrium and ATP and phosphocreatine reached a steady-state-concentration in the synaptosomes. The AMP deaminase activity localized in the synaptosomes (Jansson & Härkönen to be published) would explain the decreasing sum of adenylates during incubation.

Energy metabolism and synaptic function

The function of nerve tissue, especially synaptic transmission (Larrabee & Klingman 196., Harkönen et al. 1969) is very sensitive to glucose deprivation. It has been shown that failure of transmission occurs long before ATP and phosphocreatine are exhausted in the ganglion, and this seems to coincide with depletion of hexoses (Harkonen et al. 1969). Evidence also indicates that the locus of the failure is at the synaptic level and several explanations have been offered without direct experimental support (see Larrabee & Klingman 1962, Nicolescu et al. 1966, Harkonen et al 1969) The present results show that 2 h after the start of incubation (when transmission through the ganglion is known to fail Harkonen et al. 1969) the synaptosomes were depleted of ATP and phosphocreatine, although glycogen was still present. Therefore, blockage of impulse propagation at the synaptic level appears to be due to local depletion of ATP in the nerve endings, followed by failure of the ion pump

The other possible explanation would be the catabolism of tissue elements in the absence of glucose; this hypothesis gains support from the high production of ammonia and disappearance of vesicles containing the transmitter It might well be that failure of chemical transmission is due to the two phenomena together The earlier hypothesis of depletion of acetyl-CoA, suggested because failure of transmission coincides with depletion of hexoses, seems no longer tenable (Härkonen et al. 1969)

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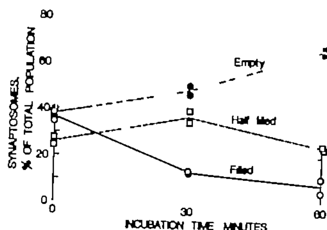


Fig 6 The effect of incubation at 37°C in oxygenated electrolyte solution without added energy substrates on the preservation of synaptosomes The "filled" synaptosomes contain the normal quantity of vesicles (occupying 60–100% of the synaptosome area) those which are "half filled" have lost about half their vesicles and the "empty" synaptosomes contain few if any vesicles (occupying at most 20% of the synaptsome area) The points represent the result of two independent investigators.

PO_4^{3-} During the first 15 min 50% of the ATP and phosphocreatine were lost but after this initial fall there was no further loss of either compound

Ammonia production and vesicle preservation in incubated synaptosomes

For synaptosomes incubated in an oxygenated substrate free medium ammonia production was after an initial lag about 1 5 mmol/kg prot./min during the first 30 min and then levelled off (Fig. 5) Under nitrogen ammonia production was much slower averaging 0 4 mmol/kg prot /mm (Fig. 5)

Synaptosomes incubated at 37°C without substrates showed clear-cut morphological alterations as compared with unincubated synaptosomes stored at 4°C on ice The number of vesicles decreased drastically and often enlarged vesicles as well as swollen intrasynaptosomal mitochondria could be seen These changes were apparent after as little as 30 min but after incubation for 1 h most synaptosomes contained hardly any intact vesicles and were filled with debris These changes were evaluated semi-quantitatively (see Material and methods) At zero time well preserved synaptosomes and synaptosomes with a very low vesicular content were about equal in number (Fig 6) However after 1 h at 37°C only about 10% of the synaptosomes were in good condition and nearly 70% were badly damaged (empty of vesicles). When performed in the absence or presence of oxygen incubation had the same morphological effect

DISCUSSION

Pattern of utilization of energy reserves

Despite the low concentration of endogenous substrates the metabolic changes that occurred when the tissue was incubated in an oxygen or nitrogen atmosphere show that the synaptosomes are [illegible] and utilize their energy reserves according to the requirements In both conditions glycogen [illegible] and phosphocreatine the major energy-yielding compounds in nervous tissue (besides glucose which in vivo originates from blood), were consumed in a pattern quite different from that seen in immature nerve cells (Hemminki & Härkönen 197[illegible] or in the sympathetic ganglion (Härkönen et al 1969) In synaptosomes ATP and phosphocreatine were used rapidly in both the presence and the absence of oxygen while in the other tissues they lasted much longer especially in oxygen. The [illegible] initial decline of glycogen in synaptosomes as compared to ganglion cells or in immature nerve cells could be due to the fact that they are practically devoid of endogenous glucose which is the substrate utilized initially at least in the ganglion [illegible] most of our synaptosome preparations, glycogen was detectable at substantial concentrations [illegible] after ATP and phosphocreatine were used up [illegible] obvious from the small decrease in glycogen [illegible] 15 min in oxygen that this is not the main substrate supporting the substantial respiration. However [illegible] considerable amount of ammonia was produced between 15 and 30 min after the start of incubation [illegible] would explain the oxygen consumption, because most of the ammonia formed in nervous tissue during incubation in vitro originates from amino acids that are oxidatively deaminated (Weil-Malherbe 1962)

Oxygen consumption of isolated nerve endings

It has been shown previously that synaptosomes have an adequate endogenous respiration rate which can be stimulated by glucose or pyruvate (Bradford 1969 Verity 1972, Diamond & Fishman 1973 Bradford et al 1978) With our technique the endogenous respiration rate was higher than [illegible] manometric techniques (Bradford 1969) but [illegible]

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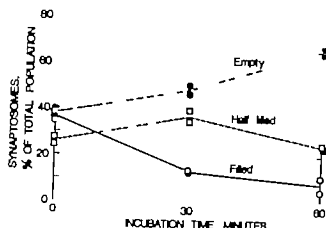


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yptophylglycine dipeptide in ACTH/MSH cells the human hypophysis its identification ıd studies on its antinociceptive effects in mice

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PARTANEN S., KAAKKOLA, S. & KÄÄRIÄINEN I. Tryptophylglycine dipeptide in ACTH/MSH cells of the human hypophysis: its identification and studies on its antinociceptiv effects in mice. Acta Physiol Scand 1979 107 213–218. Received 26 Feb. 1979 ISSN 0001-6772. Unit of Pathology Jorvi Hospital, Espoo and Department of Pharmacology University of Helsinki, Finland.

The ACTH/MSH cells of the pars distalis and pars intermedia of the mammalian hypophysis contain peptides with amino-terminal tryptophan which exhibit a strong fluorescence after treatment with modified formaldehyde vapour methods and with glyoxylic acid in the tissue sections from freeze-dried specimens. By homogenization of the hypophyses in ethanolic glyoxylic acid and subsequent heating the peptides can be con erted to highly fluorescent β-carboline derivatives, these can then be extracted with glacial acetic acid, separated by silica gel thin-layer chromatography and identified in UV light. Amino-terminal tryptophyl peptide from adult human hypophysis extracted and treated in this way gave the structure L-tryptophylglycine after acid hydrolysis. This structure was subsequently confirmed by producing a fluorescent derivativ from authentic L-tryptophylglycine using the same reaction conditions as for the tissue homogenate. This derivative moved in the same way in thin-layer chromatography as fluorescent amino-terminal tryptophyl peptide extracted from human hypophyses. Thereafter study was made of the antinociceptive effects of authentic L-tryptophylglycine administered subcutaneously in mice both alone and together with morphine -tryptophylglycine had no antinociceptive effects alone and neither did it change morphine antinociception. Also it had no apparent effects on the behaviour of mice Thus, ACTH/MSH cells contain dipeptide whose physiological function differs from the effects of ACTH MSH and endorphins.

Key-words Antinociception, extraction, hypophysis, tryptophyl peptide

The hormone storage granules of ACTH/MSH cells in both the pars distalis and the pars intermedia of all mammals studied contain substance(s) which exhibits a strong specific fluorescence after treatment with modified formaldehyde fluorescence histochemical methods and after treatment with glyoxylic acid (see Partanen 1978a). Previously this substance was thought to be serotonin primary catecholamine tryptamine or a tryptamine-like compound, but according to Sephadex G-25 chromatographic studies by Håkansson et al (1972, 1974) it is a polypeptide or polypeptides with amino-terminal tryptophan. The hypophyseal fluorogenic peptide can be demonstrated by immersion of freeze-dried hypophyseal sections in an ethanolic solution of glyoxylic acid followed by heating leading to formation of highly fluorescent dihydro-β-carboline derivatives (Partanen 1975). In model experiments glyoxylic acid treatment also appeared to induce the formation of a highly fluorescent product from amino-terminal tryptophyl dipeptide without breaking the peptide bond (Partanen 1976)

Following the same reaction principle, when rat hypophyses were homogenized and heated in glyoxylic acid-ethanol a strongly fluorescent substance could thereafter be extracted from the precipitate and separated by silica gel thin-layer chromatography (Partanen 1978b).

In the present work a study was made of the nature of this hypophyseal fluorogenic tryptophyl peptide extracted from human hypophysis. After its identification as L-tryptophylglycine research was carried out on its opioid-like effects in vivo in mice.

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Tryptophylglycine dipeptide in ACTH/MSH cells of the human hypophysis Its identification and studies on its antinociceptive effects in mice

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In the present work a study was made of the nature of this hypophyseal fluorogenic tryptophyl peptide extracted from human hypophysis After its identification as L-tryptophylglycine research was carried out on its opioid-like effects in vivo in mice.



Fig 1 The pars distalis of the hypophysis obtained within 48 h after death from a cadaver stored at 4°C Yellow cytoplasmic fluorescence in some pars distalis cells induced by immersion of freeze-dried deparaffinized sections in 4% glyoxylic acid monohydrate in ethanol at 22°C for 5 min followed by heating at 100°C for 5 min. ×200

The project was stimulated by recent findings of substances of hypophyseal origin possessing strong opioid-like effects

MATERIALS AND METHODS

Source of human hypophyse Six human macroscopically normal hypophyses were obtained at autopsy from adult patients within 48 h after death The cadavers were stored at 4°C The hypophyses were stored after dissection at −50°C.

Fl orescence histochemistry Two hypophyses were freeze-dried and embedded in paraffin wax w thout formaldehyde vapour treatment. The deparaffinized sections were briefly rinsed in absolute ethanol and immersed in 4% glyoxylic acid monohydrate (Sigma) in absolute ethanol for 5 min at 22°C blotted dry and heated at 100°C for 5 min and then embedded in Diatex (AB Wilh. Becker) (Partanen 1975) The sections were examined and photographed with a Leitz Orthoplan fluorescence microscope equipped with an HBO 200 high pressure mercury lamp (Osram) as UV light source the primary filters were BG 38, BG 3 and UG 1 and the secondary filter was K 470

Extraction of hypophyseal fluorog nic peptide Four hypophyses (wet weight ..1 g) were homogenized at °C with a glass homogenizer in 5 volumes of 4 glyoxylic acid monohydrate (GA) in ethanol the homogenate was transferred to a glass tube, hich was stoppered and heated at 65°C for 1 h After cooling to 2° the homogenate was centrifuged for 15 min at a speed of 3000 The precipitate was washed with 2 volumes of GA-ethanol recentrifuged The precipitate was washed 3 times 5 min with 3 volumes of diethylether and extracted min with 3 volumes of glacial acetic acid 1978*b*) The extract was allowed to evaporate volume and the volume was finally adjusted with glacial acetic acid to 500 μl

Thin-layer chromatography Ascending thin-layer chromatography was carried out on precoated plates without a fluorescence indicator (layer thickness 0.25 mm, Silica gel 60 E. Merck). All solvents were analytical grade The following solvent systems were used, 1) *n*-butanol–glacial acetic acid–water (4 1 1) 2) *n*-butanol–ethanol–glacial acetic acid–water (2 1 1 3) isopropanol–water (4 1), 4) *n*-butanol–glacial acid–water (4 1 1) 5) *n*-butanol–glacial acetic acid (5 1 4 upper phase), 6) *n*-butanol–acetone–water NH (15 15 5 1) The fluorescence on the plates studied with excitation light at 370 nm and photographed through a secondary cut-off filter (460 nm). Ninhydrin reagent (0 1% ninhydrin E. Merck) in *n*-butanol-glacial acetic acid (9 1) or 0.1% amido black (E. Merck) in acetic acid was used to visualize the chromatographed extract and the reference amino acids.

Acid hydrolysis The fluorescent bands separated from hypophyseal extract in thin-layer chromatography in solvent system 1 were scraped off and extracted at 22°C with 400 μl of glacial acetic acid for 30 min and with 200 μl for min. As a control the same area as above was scraped off from the pure plate and treated in the same way. The glacial acetic acid extracts were allowed to evaporate to dryness and dissolved in 0.5 ml of 6 N HCl, transferred to glass tubes filled with N_2, stopped and heated at °C for 26 h or 72 h. The hydrolysates were then evaporated to dryness and dissolved in 90 μl of 0.1 N HCl Amino acids (United States Biochemical Corporation) dissolved in N HCl (1 mg/ml) were used as a reference. They were pipetted in amounts of 1 μl (1 μg) thereafter the plates were aerated for 30 min and chromatographed.

Fluore cence model xperiments 1) L Tryptophan solution 0.5 ml (1 mg/ml dissolved in 0 1 N HCl) and of 4% GA-ethanol were heated at 65°C for 1 h, a reaction producing highly fluorescent β-carbolines (Björklund et al 1972) 3 μl of this solution, which turned yellow was used as a reference substance for hypophyseal fluorogenic substance and for its hydrolysate) Small amounts of pure L tryptophan and L tryptophylglycine (Sigma) were treated in the same way as the hypophyses, and glacial acetic extract (coloured yellow) were used in amounts of 2–3 μl as a reference for hypophyseal extract and for its hydrolysate

M asur ment of antinociception In the antinociceptive experiments male white mice of the NMRI strain weighing 36–40 g were used They were kept on a standard diet and given tap water ad libitum. The nociceptive responses were tested using the hot plate m Eddy & Leimbach

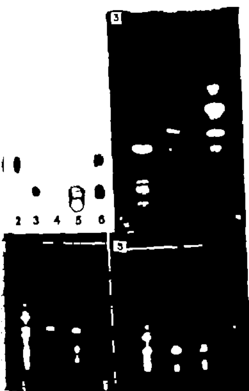


Fig. 2. A silica gel chromatograph of 15 μl of acid hydrolysate of hypophyseal fluorescent band (5) developed in n-butanol–acetone–water–25% NH_3 (15 15 15 1 v/v) and sprayed with ninhydrin. 4 control acid hydrolysate of pure silica gel plate treated in the same way as the fluorescent hypophyseal band. Glycine is seen in the acid hydrolysate of the fluorescent band. 1 Pro. 2, Ser 3 Gly 6, Pro+Ser+Gly

Fig. 3 A fluorescence photomicrograph of silica gel thin layer chromatograph of original hypophyseal extract, which was homogenized in 4% ethanolic glyoxylic acid (left), of acid hydrolysate of hypophyseal fluorescent band (centre) and of L-tryptophan (right) treated with glyoxylic acid. Solvent system n-butanol–glacial acetic acid–water (4 1 1 v/v) One strongly yellow fluorescent band seen in the *original hypophyseal extract*, two weak yellow fluorescent bands are seen near the origin. Acid hydrolysis of the strongly fluorescent band produces an additional blue fluorescent band, which moves in the same way as the blue fluorescent band of L-tryptophan treated with glyoxylic acid

Fig. 4 5 Fluorescence photomicrographs of silica gel thin layer chromatograph of the original hypophyseal extract (centre), of L-tryptophan (left) and of L-tryptophylglycine (right) treated and extracted in exactly the same way as hypophyseal homogenate. The hypophyseal fluorescent band moves in the same way as the fluorescent derivative of L-tryptophylglycine and in both the colour of fluorescence is yellow Although these bands move in the same way as the fluorescent derivative of L-tryptophan, tryptophan gives different pattern of fluorescent bands, and some fluoresce bluishly Fig. 4 solvent system as in Fig. 3. Fig. 5 solvent system n-butanol–ethanol–glacial acetic acid–water (2 1 1 1 v/v).

1953). All injections were given s.c. in doses of 0 1 ml/10 g. The substances used were L-tryptophylglycine and (−)morphine hydrochloride (Pharmacopoea Nordica). They were dissolved in 0.9% NaCl solution. The doses of morphine given refer to the base. Student's *t*-test was used in the statistical treatment of the results.

RESULTS

Fluorescence histochemistry In the hypophyseal sections treated with GA-ethanol numerous oval to round cells in the pars distalis and the invading cells of the pars nervosa exhibited moderate to strong yellow fluorescence restricted to the cytoplasm (Fig. 1)

Thin-layer chromatography of hypophyseal extract Two chromatographic plates were given an application of 200 μl of hypophyseal extract as one band 18 cm in length. The plates were developed in solvent system 1 In UV light one strongly yellow fluorescent band (corresponding to 46.7 mg of hypophyseal wet weight/1 cm) was seen In addition, one strongly fluorescent band or spot was observed when the extract (3–20 μl) was chromatographed in solvent systems 2, 3 4 and 6 (Figs. 3–5) The fluorescent spots did not stain with ninhydrin but did stain weakly with amido black. A chromatographed strongly fluorescent band was scraped off from the 200 μl extracts and extracted with glacial acetic acid From the new extract, 360 μl was evaporated and subjected to acid hydrolysis. The hydrolysis times were 26 and 72 h. Twenty μl of extract were also papetted as a band (10 mm) onto chromatographic plates and the plates were developed in solvent systems 2 and 3 One strongly fluorescent band was seen in UV light the R_f values were 0.33 and 0.61 respectively The spots did not stain with ninhydrin and no stained bands were seen on the plates.

Thin-layer chromatography of acid hydrolysate Five μl of acid hydrolysate (corresponding to 42 mg of original hypophyseal wet weight) was applied as one spot together with the control hydrolysate of pure silica gel and with reference amino acids, and developed in solvent system 4 The front was allowed to move 118 mm, and the following R_f values were obtained after visualization of amino acids



Fig 1 The pars distalis of the hypophysis obtained within 48 h after death from a cadaver stored at 4°C. Yellow cytoplasmic fluorescence in some pars distalis cells induced by immersion of freeze-dried deparaffinized sections in 4% glyoxylic acid monohydrate in ethanol at 22°C for 5 min followed by heating at 100°C for 5 min ×700

The project was stimulated by recent findings of substances of hypophyseal origin possessing strong opioid like effects.

MATERIALS AND METHODS

Source of human hypophyse Six human macroscopically normal hypophyses were obtained at autopsy from adult patients within 48 h after death The cadavers were stored at 4°C. The hypophyses were stored after dissection at −50°C

Fluorescence histochemistry Two hypophyses were freeze-dried and embedded in paraffin wax without formaldehyde vapour treatment. The deparaffinized section were briefly rinsed in absolute ethanol and immersed in 4% glyoxylic acid monohydrate (Sigma) in absolute ethanol for 5 min at 22°C blotted dry and heated at 100°C for 5 min and then embedded in Diatex (AB Wilh. Becker) (Partanen 1975). The sections were examined and photographed with a Leitz Orthoplan fluorescence microscope equipped with an HBO 200 high pressure mercury lamp (Osram) as UV light source the primary filters were BG 38, BG 3 and UG 1 and the secondary filter was K 470

Extraction of hypophyseal fluorogenic peptide Four hypophyses (wet weight ~1 g) were homogenized with a glass homogenizor in 5 volumes of 4% glyoxylic acid monohydrate (GA) in ethanol; the homogenate transferred to a glass tube, which was stoppered heated at 65°C for 1 h. After cooling to ... the homogenate was centrifuged for 15 min at a speed of 3000 r ... precipitate was washed with volumes of GA-ethanol recentrifuged. The precipitate was washed 3 times with 3 volumes of diethylether and extracted ... min with 3 volumes of glacial acetic acid ... 1978*b*) The extract was allowed to evaporate ... volume and the volume was finally adjusted with glacial acetic acid to 500 µl

Thin-layer chromatography Ascending thin-layer chromatography was carried out on precoated ... plates without a fluorescence indicator (layer ... 0.25 mm, Silica gel 60 E. Merck). All solvents ... analytical grade The following solvent systems ... used 1) *n*-butanol–glacial acetic acid–water (4 1 1 ... *n*-butanol–ethanol–glacial acetic acid–water (... 3) isopropanol–water (4 1), 4) *n*-butanol–glacial ... acid–water (4 1 1), 5) *n*-butanol–glacial acetic acid ... (5 1 4 upper phase), 6) *n*-butanol–acetone–water ... NH (15 15 5 1) The fluorescence in the plates ... studied with excitation light at 370 nm, and photographed through a secondary cut-off filter (460 nm). ... reagent (0.1% ninhydrin, E. Merck) in ... -butanol–glacial acetic acid (9 1) or 0.1% amido black (E. Merck) ... acetic acid was used to visualize the chromatographed extract and the ... erence amino acids.

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Measurement of ... ... In the antinociceptive experiments male white mice of the NMRI strain ... 36–40 g were used. They were kept on a standard diet and given tap water ad libitum. The nociceptive responses were tested using the hot plate method (Eddy & Leimbach

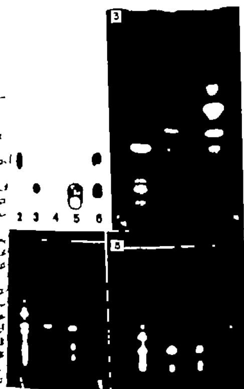


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1953) All injections were given s.c. in volume of 0.1 ml/10 g. The substances used were L-tryptophylglycine and (−)morphine hydrochloride (Pharmacopoea Nordica). They were dissolved in 0.9% NaCl solution. The doses of morphine given refer to the base. Student' *t*-test was used in the statistical treatment of the results.

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Table 1 *The effect of L tryptophylglycine on the reaction times of mice in the hot plate test (in seconds ± S E)*

Saline or L-tryptophylglycine were injected s.c 5–9 mice in each group The values of L-tryptophylglycine group are not significantly different from those of corresponding saline group

Time after injection (min)	Treatment			
	Saline	L-tryptophylglycine		
		1 mg/kg	10 mg/kg	100 mg/kg
10	7 8±0 9	8.2±0 6	–	–
20	7 8±0.7	9 1±0.7	7 6±0 4	6.5±0 6
30	7 8±0 7	8 8±0 4	–	–
40	8.4±0 7	8.0±0.5	–	–

with ninhydrin. Ser 0 21 Phe 0.53 Asp 0 18 Val 0 42 Tyr 0 49 Glu 0 ?4 Arg 0 08 CySH 0 07 Trp 0 55 His 0 07 Thr 0.27 Met 0 45 CyS-SCy 0 07 Lys 0 06 Ala 0 28 Ile 0 51 Asn 0 18 Pro 0 21 Gly 0.22 Leu 0 53 and the acid hydrolysate of fluorescent band 0 21 The colour was violet as in the serine and glycine spots thus differing from the proline spot which was yellow The hydrolysate of pure silica gel plate did not give any definite spot The results were the same after 26 and 7? h of hydrolysis Thus the conclusion was drawn that hypophyseal amino-terminal tryptophyl peptide contains either Ser or Gly or Pro or some combination of these amino acids Thereafter acid hydrolysate from a fluorescent band was developed in several solvent systems and only in system 6 did Ser and Gly move clearly separately Pro and Gly also moved separately (Fig ?) Glycine was thus identified in the hydrolysate

Thin layer chromatography of fluorescent bands When portions of acid hydrolysate of hypophyseal extract (?.5–?0 μl) were chromatographed in the solvent systems described two fluorescent spots or bands were seen in all except solvent system 3. One moved as a hypophyseal fluorescent band, and the spots were yellow fluorescent the second moved as one fluorescent spot or band of GA-tryptophan mixture and the spots were bluish fluorescent (Fig 3) Taking together the observations of the presence of both amino-terminal tryptophan and glycine in the hypophyseal fluorogenic peptide the original hypophyseal extract and products of L-tryptophan and L tryptophylglycine treated in the same way as the tissue extract were chromatographed in solvent systems ? and 4 The hypophyseal fluorogenic substance moved in the same way as L-tryptophylglycine and both fluoresced yellow In L-tryptophan extract several fluorescent spots were observed which moved in a slightly different way in addition blue fluorescent spots were produced (Figs 4 5) The present results gave L-tryptophylglycine for the structure of the hypophyseal fluorogenic peptide and its antinociceptive effects were thereafter studied

Table 2. *The effect of L tryptophylglycine on the antinociceptive effect of morphine in the hot plate test in mice*

The second injection was given 5 min after the first one all the injections were given s c. The reaction time was measured 20 min after the first injection. *n* = number of animals. * $P<0.001$ compared to the saline + saline group The values of L-tryptophylglycine + morphine group are not significantly different from those of saline + morphine group

Treatment (mg/kg)	*n*	Reaction time (seconds ± S E.)
Saline + saline	10	7.8±0.5
Saline + morphine (15)	10	13 9±1 0*
L Tryptophylglycine (10) + morphine (15)	1	1 8±0 9
Saline + morphine (30)	7	46.7±3 4
L Tryptophylglycine (1) + morphine (30)	7	39 9±4 4
L Tryptophylglycine (10) + morphine (30)	10	45 4±3 2

L-Tryptophylglycine and testing of antinociception. L-Tryptophylglycine alone even at high doses, had no significant antinociceptive effects measured by the hot-plate method (Table 1). Moreover at doses of 15 and 30 mg/kg significantly prolonged the hot-plate time but L-tryptophylglycine did not change the antinociceptive effect of morphine (Table 2). L-Tryptophylglycine did not have any clear effects on the behaviour of mice at the doses used.

DISCUSSION

The yellow cytoplasmic fluorescence in certain hypophyseal cells induced by GA-ethanol indicated that a fluorogenic substance remains unaltered in histochemical reactivity when hypophyses are obtained than 48 h after death from cadavers stored at 4°C. Because other hypophyseal cells did not exhibit any specific fluorescence it seems justifiable to assume that the fluorogenic substance obtained with GA ethanol treatment of hypophyseal extract is the same as that demonstrated histochemically. In previous works this fluorogenic substance has been localized in hormone storage granules of ACTH/MSH cells of the mammalian hypophysis with both histochemical and biochemical separation methods (Håkanson et al. 1975, Partanen 1978*a*).

The present results gave the structure L-tryptophylglycine for the hypophyseal fluorogenic substance. The molecular weight of this dipeptide is 261 and differs from the previously reported molecular weight (over 5000) of the main fraction of amino-terminal tryptophyl peptides separated by Sephadex G 25 chromatography from hypophysis and various parts of the central nervous system although the presence of smaller amino-terminal tryptophyl peptides was not excluded (Håkanson et al. 1972, Edvinsson et al. 1973, Håkanson et al. 1974). In addition, the extraction methods used by Håkanson and co-workers (Håkanson et al. 1972, Edvinsson et al. 1973, Håkanson et al. 1974) and by the present authors differed which could explain the differences concerning the molecular weight.

It has been demonstrated fluorescence histochemically that amino-terminal tryptophyl peptides of the hypophyseal cells decreased concomitantly with a decrease in the immunohistochemically demonstrable ACTH in hamster pars distalis (Håkanson & Sundler 1977) and in the number of electron microscopically demonstrable hormone storage granules in the pars intermedia (Partanen & Back, to be published). Thus, amino-terminal tryptophyl peptides are released into the peripheral circulation and possibly also directly into the central nervous system via the retrograde hypophyseal portal circulation (Oliver et al. 1977) together with other hormones of ACTH/MSH cells. It has been shown that β-endorphin shows parallel changes both in blood and in the hypophysis with ACTH (Guillemin et al. 1977). The sequence Try-Gly is present in β-lipotropin, ACTH and MSHs but it has no ACTH or MSH-like activity which requires at least a tetrapeptide in which Try-Gly is in the carboxyl terminal position (Wallis 1975). In addition, the present results showed that L-tryptophylglycine alone administered subcutaneously into mice had no antinociceptive effects and no influence on morphine antinociception even at high doses. The content of amino-terminal tryptophyl peptides in the mouse hypophysis expressed in the form of tryptophylalanine equivalents has been reported to be about 10 μg/g of wet weight (Håkanson et al. 1974). Thus, although it is possible that L-tryptophylglycine is hydrolyzed by peptidases as enkephalins if given systematically (Pert et al. 1976) the doses were so large that intact tryptophylglycine was probably present in the circulation at times when the measurements were made. Also it seems that L-tryptophylglycine does not exert any physiological effect in its hydrolysis to tryptophan which subsequently leads to increased serotonin synthesis in the central nervous system (Fernstrom & Wurtman 1972). Serotonin has been shown to modify the effects of opiates, and it has been suggested that opiates exert their effect by changing the serotonin content (Hosobuchi et al. 1977). Further L-tryptophylglycine had no apparent effect on the behaviour of mice. Thus, the physiological significance of L-tryptophylglycine seems to be different from the effects of ACTH, MSH and hypophyseal opiate-like peptides. It might be a physiologically inactive part of a larger molecule or a dipeptide with its own unknown function.

This work was supported by grants from E. Aaltonen Foundation and J. K. Paasikivi Foundation.

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Comparison of effects of pentobarbital and ethanol on the neuronal activity in the posterior parietal association cortex

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HYVÄRINEN J LAAKSO M., ROINE, R. & LEINONEN L.. Comparison of effects of pentobarbital and ethanol on the neuronal activity in the posterior parietal association cortex. Acta Physiol Scand 1979 107 219-225 Received 28 Febr 1979 ISSN 0001-6772. Department of Physiology University of Helsinki, Finland.

The problem of this study was whether the effects produced by alcohol in the posterior parietal association cortex are specific to this drug or shared by other centrally acting depressant drugs such as barbiturates. The effect of graded doses of pentobarbital on multineuronal impulse activity was recorded with transdural microelectrode technique in 30 expts. in Brodmann' area 7 of five stump-tailed monkeys (Macaca speciosa). The results were compared with those from 32 expts. performed with alcohol and published separately The dosage of the two drugs was determined on the basis of the monkeys sensori-motor coordination which was assessed with rating scale of reaching accuracy for food rewards. There were several recording sites where the actions of the two drugs were similar at similar behavioural levels of intoxication. However in the distribution of effects among various functional types of recording sites significant difference was found between pentobarbital and alcohol Alcohol commonly diminished cellular activity related to motor behaviour (reaching, grasping) and only rarely responses to somesthetic stimuli, whereas the effects of pentobarbital were the opposite being most common on somatosensory responses and least common on activity related to motor behaviour Also responses to visual stimuli were more sensitive to pentobarbital than to alcohol. The actions of pentobarbital and alcohol on responses evoked by sensory stimulation differed significantly ($P<0.01$) W conclude that significant differences exist in the mechanisms of action of alcohol and barbiturate on the associative ystems of the brain.

Key ords Ethanol, pentobarbital, parietal association cortex, multineuronal activity

It has been suggested that the effects observed in the CNS after administration of alcohol are nonspecific and similar to the effects produced by other centrally acting depressant drugs such as barbiturates (Kalant 1975) With EEG and evoked potential techniques it has been demonstrated that cortical associative zones and midbrain reticular formation are sensitive to the actions of alcohol (DiPerri et al. 1968, Perrin et al 1974). W have shown previously that the cellular activity in the posterior parietal association cortex is sensitive to the action of alcohol (Hyvarinen et al. 1978), and that often the behavioural action of alcohol is closely paralleled by its action of the cellular activity in this cortical region that normally participates in the sensorimotor control of hand- and eye-movements (Hyvarinen & Poranen 1974 Mountcastle et al. 1975) It was therefore of interest to study whether the effects produced by alcohol in the posterior parietal association cortex are specific to alcohol or whether barbiturates would produce similar results. We performed this study using pentobarbital in doses that produced effects in the monkeys sensori-motor coordination comparable to those produced by alcohol. The results indicate that although similarities exist in the actions of these two drugs there are also significant differences.

MATERIAL AND METHODS

This study was performed with 5 Macaca speciosa monkeys weighing 6 to 10 kg. It was not possible to hold single units long enough for the study of the drugs, because the

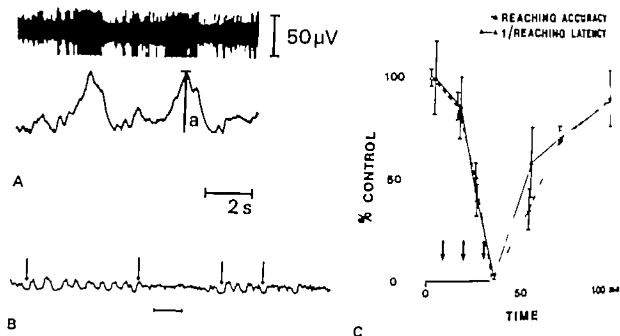


Fig 1 (A) Multineuronal impulse activity (*upper trace*) and the same activity rectified and integrated (*lower trace*). The control responses during reaching with ipsilateral arm toward a food reward are seen. Measurement of the amplitude of an integrated response from the base-line of the recording is indicated (a) Mean amplitudes and their standard deviation were calculated of ten consecutive responses. The mean and standard deviation values obtained during the action of the drug were then expressed as percentage of the mean control value (B) Spontaneous activity in an integrated multineuronal recording showing two periods of rhythmic discharge The beginning and the end of the rhythmic periods are indicated by arrows Pentobarbital was injected 12 5 mg/kg divided in 4 doses the sample was recorded in the recovery phase 53 min after the last dose. For measurement of the percentage of time of rhythmic discharge 40 s of spontaneous activity was recorded the duration of rhythmic periods was measured and expressed as percentage of the total duration of the recording (C) The effect of pentobarbital on the reaching accuracy score and the inverse value of the reaching latency measured electrically At each arrow 4 mg/kg of pentobarbital was given through the intracardial catheter. Mean and standard deviation values of 10 trials expressed as per cent of control values. The same notation is used in later figures

induction of and recovery from the drug effects took several hours. Therefore multiple unit recordings were made Multineuronal impulse activity was recorded in the same part of Brodmann's area 7 as in our previous paper (Hyvarinen et al 1978) in chronic expts with movable microelectrodes. No other anesthesia except the drugs studied was used during the expts The recording methods have previously been described in detail (Hyvarinen & Poranen 1974 Hyvarinen et al. 1978 1979) In this study multineuronal activity was recorded with glass-insulated tungsten electrodes moved into the cortex through the intact dura with a hydraulic micromanipulator The multineuronal activity (Fig. 1 A) was filtered rectified and integrated using the apparatus described by Weber & Buchwald (1965). High-pass filtering at 300 500 or 1000 Hz eliminated completely slow wave signals from the recording leaving only action potential spikes Judging from the variance in the amplitudes of the action potentials recorded these were discharged by approximately 5-10 neurons. Many things influence the amplitude of the multineuronal responses, such factors as the size of the electrode, the size of the neurons recorded and movements of the electrode in reference to the cells studied. To eliminate the influence of these variables, the electrodes were not moved during the study of the drugs and the amplitudes of the integrated multineuronal responses were expressed as percentages of the amplitudes in the control recordings made prior to the administration of the drug (Fig 1 A) The drugs were infused through an intracardial catheter permanently implanted in the right atrium of the heart as previously described (Hyvarinen et al. 1978). In some expts pentobarbital (Nembutal R) was given i.m.

30 expts. were performed using pentobarbital and the results of these expts. were compared with those from expts performed with alcohol and published previously (Hyvarinen et al 1978) The dosage of the drugs was adjusted by following the behavioural action. For this purpose a rating scale was constructed for estimation of the monkey's reaching accuracy for food reward. Ten points were given for normal rapid and accurate reaching for food rewards offered by the experimenter. With increasing dosage of the drugs the reaching became slow

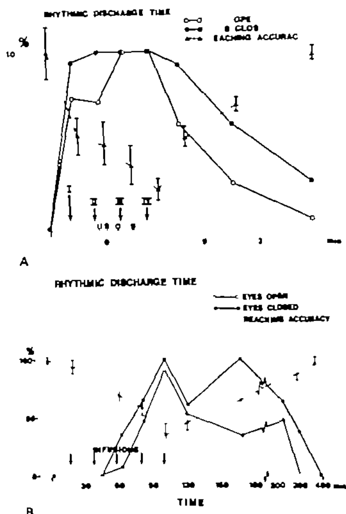


Fig 2 The development of the rhythm during the action of the drug. The percentage of recording time when the spontaneous discharge was rhythmic is indicated together with the reaching accuracy (A) Pentobarbital infusions (*arrow*) of 5 (*first arrow*) +3 2.5 mg/kg (B) Alcohol infusions 5 0 5 g/kg More rhythm was present when the monkey s eyes were closed

and inaccurate and finally ceased altogether Zero points were given for no attempt to reach and intermediate values were given for intermediate accuracies and speeds Three observers used to do these scorings getting consistent results. For one drug level their estimates did not differ from each other more than ±10% In some expts the adequacy of this arbitrary rating scale was compared ith electrical measurements of the monkey reaching latency using specially constructed food well. A close correlation as observed between the reaching scores and the inverse values of reaching latency (Fig. 1C) The appearance of rhythm in the spontaneous multineuronal discharge as studied by measuring the time that the discharge was rhythmic. This time was expressed as per cent of total recording time as indicated in Fig. 1B At least 3 successive waves were required for period to be considered rhythmic. The dominant frequency of the rhythm was determined by counting the number of distinct waves per time unit during the rhythmic periods.

RESULTS

Both pentobarbital and alcohol produced a rhythmic spontaneous multineuronal discharge

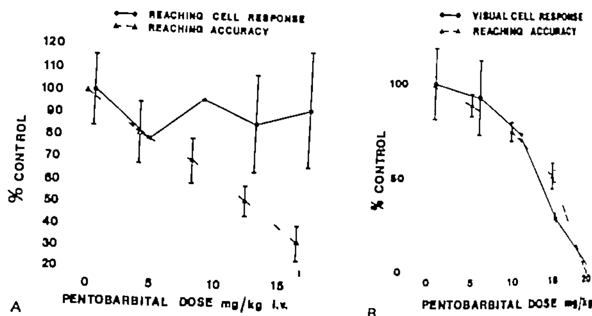


Fig 3 (A) Lack of effect of pentobarbital on the amplitude of integrated responses at a recording site responding to reaching movements performed with the contralateral hand. Although the reaching accuracy fell with increasing dose of pentobarbital the cellular responses remained unaffected Means and standard deviations of 10 responses. (B) The effect of pentobarbital dose on the amplitude of a visual response and reaching accuracy At this recording site new objects brought into the monkey s visual field close to the animal triggered a response but visual fixation of familiar food did not produce responses At each dose level 10 new objects were presented to the monkey as stimuli and the means and standard deviations of the responses are plotted in the figure

This rhythmicity appears to be related to the slow EEG rhythms produced by centrally depressant drugs In the multiple unit records the proportion of time that the spontaneous activity was rhythmic increased with increasing dose Closing of the eyes increased the prevalence of the rhythmic activity It was also evident that the amount of rhythmic discharge correlated inversely with the performance of the animal expressed as reaching accuracy and that this relationship was similar for alcohol and pentobarbital (Fig ?) In alcohol experiments the frequency of the rhythm became slower with increasing dose (Hyvärinen et al 1978) but such a relationship was not seen in pentobarbital experiments in which the dominant rhythm stayed at approximately ? Hz when present

7 expts with pentobarbital were performed at sites where the cellular activity correlated with reaching movements performed with the contralateral arm and hand as described before (Hyvärinen & Poranen 1974 Mountcastle et al 1975) The results were compared with those obtained from 1 alcohol experiments on functionally similar recording sites. The effects on motor responses were less common in the pentobarbital group in which three of eight sites were affected by the drug whereas 1 of 12 sites were sensitive to alcohol (Table 1). The negative result of one pentobarbital experiment is illustrated in Fig 3 A The differences of the effects of pentobarbital and alcohol on cellular activity related to motor behaviour were not statistically significant however

Visual stimuli activated 13 recording sites in pentobarbital experiments. Eleven of these were sensitive to the action of pentobarbital whereas two were not These expts are compared with results from 10 similar expts. performed with alcohol in 5 of which there was an effect of the drug (Table 1) These sites responded to following types of visual stimuli visual approach towards the animal visual fixation of objects, visual presentation of new objects uncovering of the eyes, and movement in the visual field

Fig. 3 B illustrates amplitudes of visual responses as a function of pentobarbital dose at a site where the cells responded to new visual stimuli. Movement of indifferent visual stimuli in the visual field of the animal or visual fixation of food did not activate this recording site The effect of pentobarbital on this response was strongly decremental

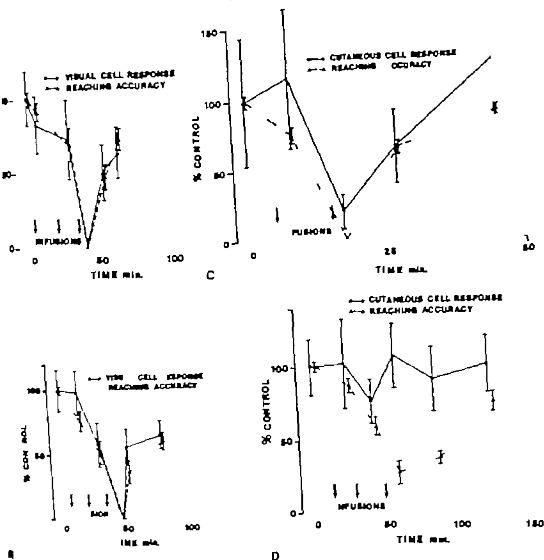


Fig. 4 *Effects of pentobarbital (A) and alcohol (B)* on the responses at site responding to large moving stimuli brought to the visual field from the contralateral side. The two experiments were performed at the same cortical site with an interval of four days. Pentobarbital infusions (*arrows*) 5 mg/kg each and alcohol infusions 1 g/kg each. Means and standard deviations of 10 responses. (C) Effect of pentobarbital on responses to cutaneous touch and on reaching accuracy. Two infusions of 5 mg/kg each are given at arrows. (D) Effect of alcohol on similar responses and reaching accuracy. Infusions of 1 g, 1 g and 0.5 g/kg were given at arrows. Means and standard deviations of 10 responses. The two experiments were performed at the same cortical site with an interval of eight days. This site responded to touch of the snout.

Fig. 4 A and B show decrement of visual responses recorded at another site during the action of pentobarbital and alcohol. At this site both drugs produced close to identical effects.

Somesthetic responses were less often affected by alcohol than by pentobarbital. Alcohol reduced the response amplitudes at only 3 of 10 recording sites (Table 1), whereas 7 of 9 recording sites responding to somesthetic stimulation were sensitive to pentobarbital. The effect of pentobarbital on the somesthetic responses was statistically significantly more common ($P<0.05$ χ^2-test) than the effect of alcohol.

Fig. 4 C and D show an example of different ef-

Table 1 *Effect of alcohol and pentobarbital on multineuronal impulse responses at different recording sites in area 7*

	Pentobarbital effect				Alcohol effect					
	Yes		No		Yes		No		Total	
Type of activity	N	%	N	%	N	%	N	%	N	
Motor (reaching grasping, manipulation)	3	37	5	63	8	67	4	33	[illegible]	[illegible]
Visual (complex visual phenomena)	11	85	2	15	5	50	5	50	[illegible]	[illegible]
Somatosensory (skin muscle joint)	7	78		[illegible]	3	30	7	70	[illegible]	[illegible]
Total	21	70	9	30	16	50	16	50	[illegible]	

The differences between the effect of alcohol and pentobarbital are statistically significant (χ^2-test) on the somatosensory responses ($P<0.05$) and on the sensory responses (somatosensory and visual combined $P<0.01$). The difference between the effects on motor responses and sensory responses is significant for pentobarbital ($P<0.05$).

fects of pentobarbital and alcohol on responses to similar cutaneous stimuli As reported previously no effect of alcohol on the cellular activity was observed in the primary somatosensory cortex (Hyvärinen et al 1978) One experiment performed with pentobarbital in S I indicated a small effect also in this region

Table 1 indicates the distribution of effects of alcohol and pentobarbital in 62 expts The level of dosage of the two drugs was comparable since both reduced the reaching accuracy close to zero The effect of pentobarbital on all sensory responses (somesthetic and visual combined) was significantly more common that the effect of alcohol ($P<0.01$ χ^2-test) On the other hand an effect of alcohol was more often observed on cellular responses related to movements although this difference was not statistically significant

DISCUSSION

The multiple unit recording method used in this study can be considered as an intermediate technique between recording of intracortical slow wave activity and single unit activity For the study of the action of drugs this recording method proved suitable as the effects of the drugs on the multiple unit activity were quite distinct To sample sufficiently single unit data for firm conclusion on the action of the drugs would have required considerably more experiments. Several factors may influence the magnitude of multiple unit activity Such factors are for instance the size of the electrode tip and the size of the cells recorded as well as the distance between the cells and the electrode. The influence of these factors was eliminated from the present study of drug effects by leaving the electrode in place prior to and during the study of the drugs and by expressing the amplitude values as percentage of the control values obtained prior to the injection of the drugs.

It has been known that sensory areas of the cortex are less sensitive to anesthetic than the association areas (Albe-Fessard & Fessard 196[illegible], Thompson et al 1963) and this fact has been applied to delineate the sensory and motor areas by the evoked potential and electrical stimulation techniques in deep pentobarbital narcosis (Woolsey et al 194[illegible], Woolsey 1958) On the other hand it has been suggested that the neural effects of alcohol are similar to those of other centrally acting depressant drugs such as barbiturates (Kalant 1975) The effects observed in this study were indeed similar in many respects For instance both drugs produced a rhythmic spontaneous activity that was similarly related to the behaviour of the animal i.e. the rhythmicity increased as the reaching accuracy decreased (Fig) Also the effects of the two drugs on cellular responses to stimuli were comparable at several recording sites in area 7

Behaviourally the actions of the two drugs appear different however Whereas small doses of pentobarbital produce a general appearance of seda-

and a tendency to fall asleep the motor per mance is not much disturbed when awake Al- l, however produces often a euforic state ring hich the motor beha iour increases but be s not incoordinated Therefore one would expect e effects of the two drugs to differ at some ural level

This work indicates that although there are many milarities in the action of alcohol and pentobarbi- l on the multineuronal impulse activity in the sterior parietal association cortex the effects also ffer from each other The differences are most ear on the sensory responses (somesthetic and sual) that are significantly more often affected by ntobarbital than by alcohol Cellular activity re ted to motor behaviour on the other hand was ore often affected by alcohol than by pentobarbi- l (Table 1) although this difference was not statis- cally significant. The difference in the distribution f effects appears consonant with the behavioural fects of the two drugs. The general sedation pro- uced by pentobarbital correlates with its depress- g action on the sensory responses in area 7 hereas the motor incoordination produced by al- ohol correlates with the effect that this drug has on e cellular discharges related to motor perform- nce.

Studies on the neural effects of alcohol performed th EEG- and evoked potential-techniques have dicated that cortical association zones and the ticular formation are more sensitive to the drug han the specific cortical projection areas (DiPerri t al. 1968, Perrin et al 1974) Consequently it has een suggested that the polysynaptic structures are more susceptible to the actions of alcohol than sen- ory systems that have simpler synaptic connec- ons (Wallgren & Barry 1970, Himwich & Callison 1972, Kalant 1975) On the basis of these results the effect of pentobarbital appears to accumulate in the successive sensory ynapses and this may be true for alcohol also (Hyvarinen et al 1978) However the lesser effect of pentobarbital on the discharges related to motor performance complicates this in- terpretation. Although the number of ynapses in a neural pathway may be important in determining the action of that kind of drugs there appear to be differences in the details of the mechanisms in- volved.

W sh to thank Mr Ilkka Linnankoski, Mrs Katriina Lauren and Mrs Raiva Karttunen for technical assistance

This work has been supported by grant from the Finnish Foundation for Alcohol Studies.

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	N	%	*N*	%	*N*	%	*N*	%	*N*	%
Motor (reaching, grasping manipulation)	3	37	5	63	8	67	4	33	20	[illegible]
Visual (complex visual phenomena)	11	85	2	15	5	50	5	50	23	[illegible]
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Total	1	70	9	30	16	50	16	50	6.	

The differences between the effect of alcohol and pentobarbital are statistically significant (χ^2-test) on the somatosensory responses ($P<0.05$) and on the sensory responses (somatosensory and visual combined $P<0.01$). The difference between the effects on motor responses and sensory responses is significant for pentobarbital ($P<0.05$).

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Behaviourally the actions of the two drugs appeared different however Whereas small doses of pentobarbital produce a general appearance of sedation

ıcrease in local cerebral blood flow induced by circulating drenaline involvement of blood brain barrier dysfunction

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ABDUL RAHMAN A. DAHLGREN N JOHANSSON B. B & SIESJÖ, B. K. Increase in local cerebral blood flow induced by circulating adrenaline: invol ement of blood-brain barrier dysfunction. Acta Physiol Scand 1979 107 227–232. Received 22 March 1979 ISSN 0001-6772. Laboratory of experimental Brain Research and Departments of Anesthesia and Neurosurgery University of Lund, Sweden.

The influence of intra enous infusion of adrenaline (8 µg kg min^{-1}) upon local cerebral blood flow (CBF) in paralyzed and artificially ventilated rats was measured autoradiographically with ^{14}C-iodoantipyrine as the diffusible tracer At this dose adrenaline invariably increased local CBF even though blood pressure was close to normal at the time of the CBF measurement. In average, local CBF increased to 400% of control. In 6 of 9 animals the increase in flow was inhomogeneous with randomingly distributed areas of ery high flow rates. Experiments with i administration of Evans blue prior to infusion of adrenaline showed that areas of Evans blue extravasation appeared in 3 of 4 animals. Although areas of extravasation often corresponded to areas of high flow rates the former were much more circumscribed. Furthermore, very high flow rates were found in areas showing no sign of blood-brain barrier dysfunction. It is concluded that the increase in CBF was at least partly due to pressure-mediated passage of adrenaline across the blood-brain barrier but that such passage can occur in the absence of macroscopically visible extravasation of protein.

Key ords. adrenaline local CBF blood-brain barrier dysfunction

During recent years, it has become increasingly evident that biologically active amines influence cerebral metabolic rate for oxygen (CMR_{O_2}) and cerebral blood flow (CBF). Thus, at least under some circumstances, intravenously or intracarotidally administered noradrenaline or isoprenaline can give increases in CMR_{O_2} and CBF (James & MacDonnel 1975 MacKenzie et al 1976a for further literature see Kuschinsky & Wahl 1978) Since cathecholamines are supposed not to penetrate the blood-brain barrier (Weil-Malherbe et al. 1961 Bertler et al 1966, Oldendorff 1971) it has been tentatively concluded that the barrier must be "opened" by increasing the blood pressure or the osmolarity of the blood perfusing the brain, before circulating amines can exert their effects on intracerebral receptors (MacKenzie et al 1976a and). However this point may not be settled since others have concluded that the balance between α (constrictory) and β (dilatory) adrenoreceptor activity in the cerebral vessels is such that a precipitous increase in local amine concentration, one that would result from an opening of the blood-brain barrier actually may transform a dilatory response to a constrictory one (McCalden et al. 1977).

Results from this laboratory have shown that CMR_{O_2} and CBF may rise appreciably in certain stressful situations and that these increases are minimized or prevented by pretreating the animals with propranolol, or by adrenalectomy (Carlsson et al 1977) Since it could be suspected that circulating catecholamines were involved the cerebral metabolic and circulatory effects of i.v infusions of noradrenaline and adrenaline were tested in lightly anesthetized rats. The results showed that nora-

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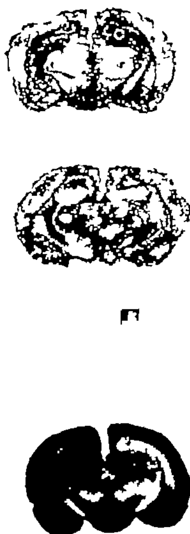


Fig. 2. Diagram illustrating effects on local CBF of hypercapnia (top) and adrenaline infusion (middle), as extravasation of Evans blue-protein complex as result of adrenaline infusion (bottom). The colour scales for l-CBF are only qualitative and not internally comparable. They therefore only illustrate the distribution of high flow (yellow – red) and low flow (green – blue). Note homogenous increase in l-CBF in hypercapnia and patchy increase after adrenaline infusion. The colour images were processed in the automatic microscanning system developed in the Laboratory of Cerebral Metabolism, National Institute of Health, by courtesy of dr L. Sokoloff.

Table 1 *Physiological parameters for the experimental groups*
The values are means ± S E.

Experimental group	Body temp (°C)	MABP mmHg Pre-infusion	MABP mmHg Max	MABP mmHg During measurement of CBF	Pa_{O_2} (mmHg)	Pa_{CO_2} (mmHg)	pH
Control (*n*=6)	37 0±0 [illegible]		148±5	14[illegible]±4	103±2	37 0±0 7	7 [illegible]
Adrenaline (*n*=9)	37.3±0 1	137±4	181±7	138±7	96±5	39.3±5	7 [illegible]

drenaline in doses of 2 5 and 8 μg kg⁻¹ min⁻¹ gave rise to a moderate (average 50%) increase in CMR_{O_2} which was blocked by propranolol However following adrenaline in a dose of 8 μg kg⁻¹ min⁻¹ CMR_{O_2} was doubled and in many animals CBF rose 4- to 5-fold (Berntman et al 1978)

Various vasoactive substances like metaramine angiotensin noradrenaline and adrenaline can induce acute arterial hypertension in experimental animals leading to blood brain barrier (BBB) dysfunction It has been shown that areas with BBB dysfunction as revealed by Evans blue or sodium fluorescein extravasation have higher CBF than non-damaged areas suggesting a local failure ("break through") of autoregulation (for review of literature see Johansson 1974*a* and 1976 MacKenzie et al 1976*b*)

The present experiments were undertaken to provide answers to the following three questions (1) Is the previously recorded increase in cortical CBF following adrenaline infusion a generalized phenomenon affecting all brain regions? (2) Is the flow homogenously increased or does it occur in a "spotty" fashion? (3) If the latter is true is the local increase in CBF correlated with signs of BBB dysfunction induced by the increase in blood pressure? In order to provide answers to these questions adrenaline was infused i v in rats in a dose of 8 μg kg min We measured local CBF with an autoradiographic technique using ¹⁴C iodoantipyrine as diffusible tracer and evaluated BBB dysfunction by recording the penetration of Evans blue from blood to tissue

METHODS

Operative procedure Male Wistar rats, weighing 320–400 g, were allowed free access to pellet food and tap water until the day of operation Anesthesia was induced with –3% halothane. The animals were then tracheotomized, immobilized with tubocurarine chloride (1.5 mg kg i) and artificially ventilated with 75% N_2O and 25% O_2. At this point, halothane supply was discontinued. Both femoral arteries and femoral veins were cannulated, the arteries to allow blood pressure recording and sampling of arterial blood for measurements of blood gases, pH and ¹⁴C iodoantipyrine activity during CBF measurement and the veins to allow infusion of adrenaline and of tracer. Body temperature was adjusted to 37°C and arterial P[illegible] to 35–40 mmHg Heparin was given i.v in a dose of [illegible] I U kg

Infusion of adrenaline Adrenaline was obtained as the bitartrate dissolved in physiological saline at a concentration of 1 mg ml (ACO Stockholm). Before infusion [illegible] ml of this stock solution was mixed with physiological saline to a final volume of 10 ml yielding an adrenaline concentration of 80 μg ml About 30 min following the completion of the operative procedures the adrenaline was infused at a rate of 8 μg kg min Local CBF was measured after an infusion period of 10 min.

Measurement of local CBF We measured local CBF with an autoradiographic technique utilizing ¹⁴C-iodoantipyrine as a tracer (Sakurada et al. 1978). Details of methodology was as described in a preceding communication (Abdul-Rahman et al. 1979). It should be emphasized that the method is of limited accuracy at very high flow rates Thus although quantitative figures have been given even for flow rates exceeding 5 ml g min these values are only approximations.

Measurement of BBB dysfunction To test the function of the blood-brain barrier (BBB) Evans blue in a dose of [illegible] ml kg was given i v as a [illegible]% solution in saline before the adrenaline infusion was started In this dose the tracer binds to serum albumin in vivo Extravasation of the dye protein complex could be macroscopically observed However in all 4 animals infused with Evans blue the brains were sectioned in a microtome and photographed In order to allow a correlation between local CBF and Evans blue extravasation alternative sections were taken for autoradiography and photography respectively

Calculation Statistical differences were calculated using Student *t* test

RESULTS

As stated adrenaline was infused i v in a dose of 8 μg kg min in 9 rats with subsequent autoradiographic determination of local CBF 10 min following the start of the infusion Physiological parameters are given in Table 1. In 4 of the animal

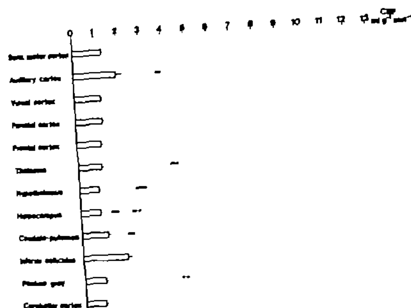


Fig. 1. Local CBF in nine animals in which adrenaline (8 μg kg⁻¹ min⁻¹) had been infused for 10 min. Control values (staples) are given as means ± S.E. In each animal, the symbols (dots) represent the average CBF within any one structure. Within each animal the average increase in l-CBF in various structures were similar and there were three animals in which very marked increases were observed throughout the brain, and others showing moderate or mild changes in l-CBF. Flow rates exceeding 5 ml g⁻¹ min⁻¹ are of limited accuracy (see Methods).

BBB dysfunction was simultaneously determined by evaluating the extravasation of administered Evans blue. In all rats infusion of adrenaline was accompanied by a rise in mean arterial pressure of 20-50 mmHg. However, as Table 1 shows the blood pressure subsequently fell and at the time of the CBF measurement, mean arterial blood pressures ranged between 120 and 170 mmHg. The table also shows that arterial P_{CO_2} was close to control values, excluding the possibility that increases in local flow rates were related to hypercapnia. A previous study (Dahlgren et al. 1979) showed that infusion of adrenaline in a dose of 8 μg kg⁻¹ min⁻¹ increased blood glucose concentration from 9 to 14 μmol g⁻¹.

The local CBF measurements gave 3 main results. First, following adrenaline infusion flow increased markedly in all brain structures. Second, in the majority of animals there were clear differences in flow rates even within the same area. Third, although 3 of 4 animals showed Evans blue extravasation marked increases in flow occurred even when no signs of protein extravasation were observed.

The first point is illustrated in Fig. 1 which gives local CBF values in each separate animal and compares them to the appropriate control values (means ± S.E., see Abdul-Rahman et al. 1979). In making this graph no attention was paid to differences in flow within a structure. Instead, an average value was obtained by making 5–7 densitometric readings from different parts of the structure in question. The results show that in many structures, the lowest CBF was twice the mean control value while the highest was 5-fold control. In most structures, the average increase in CBF was to 400% of control. In two structures (hippocampus and caudate putamen) the mean increase was only 2.5-fold, and in one (cerebellar cortex) it was 5-fold. These pronounced increases in flow were not due to increased perfusion pressure and abolished autoregulation. Thus, when the CBF values for any one structure were plotted against the mean arterial blood pressure at peak value as well as at the time of CBF measurement, no correlation between CBF and pressure was apparent (not shown). In fact, local CBF increased 3- to 4-fold even in animals that had mean arterial blood pressures below the

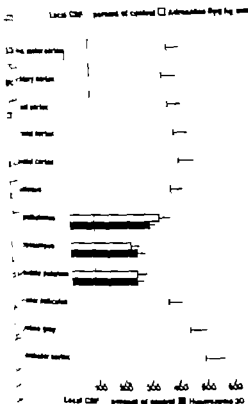


Fig. 3. Increases in local CBF in adrenaline-infused animals (unfilled staples) and in those exposed to hypercapnia (filled staples). The values are means ± S.E. in percent of normocapnic controls.

...ects, e.g. an increase in osmolarity, did not contribute to the circulatory and metabolic effects observed presently.

The magnitude of the increase in local and regional CBF following adrenaline infusion is surprising. Previous results obtained in man and in a variety of experimental animals indicate that "maximal" vasodilatation in the brain is obtained in hypercapnia (see Berntman et al. 1979). In order to allow a comparison of increases in CBF, 4 animals were exposed to marked hypercapnia (P_{CO_2} 78.9±2 mmHg) with subsequent measurement of local CBF using techniques identical to those used in the adrenaline-infused animals. Mean arterial blood pressure in these animals was 149±4 mmHg at 30 min. Fig. 3 compares local CBF in adrenaline-infused and in hypercapnic animals. Clearly, adrenaline gave rise to increases in flow that were comparable to, or even more pronounced than, those observed in hypercapnia. It should be emphasized that the infusion of adrenaline does not lead to signs of EEG activation, nor does it cause accumulation of lactic acid in the tissue (Dahlgren et al. 1979). All these results point to the fact that adrenaline exerts its effects of cerebral resistance vessels by other mechanisms rather than an activation of the reticular activation system or an accumulation of acid.

This study was supported by grants from the Swedish Medical Research Council (Project No. B79-14X-263), the Wallenberg Foundation and from U.S. PHS Grant No. 2 R01 NS-07838).

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normal mean (142±4 mmHg) at lCBF measurement

The second point is illustrated in Fig 2 which shows autoradiographs from two high flow situations In one (top) CBF was increased by raising arterial P_{CO_2} to 80 mmHg and in the other (middle) adrenaline was infused i v (This section was taken from the brain that showed the most pronounced inhomogeneities in flow) In hypercapnia local CBF in grey matter areas increased in a fairly homogenous fashion while the adrenaline infused animal showed pronounced intrastructural differences in flow For example in the parietal cortex CBF varied between 2 4 and 4 7 ml g $^{-1}$ min and in the brainstem and the hippocampus comparable differences were observed Of the 9 adrenaline-infused animals 6 showed clear intrastructural differences in CBF predominantly in parietal cortex hippocampus brain stem and cerebellum In the remaining 3 animals CBF was increased but intrastructural differences were not observed

As remarked 4 animals were used to identify areas of Evans blue extravasation In 3 of these multiple areas of extravasation were observed an example of which is shown in Fig 2 (bottom) In general areas of extravasation corresponded to CBF values that were 3- to 5-fold control However a quantitative comparison could not be made since the areas of extravasation were much more circumscribed than the areas of excessively high flow rates Furthermore equally high CBF values were observed in areas visibly devoid of extravasated Evans blue Thus although the infusion of adrenaline gave evidence of blood-brain barrier dysfunction in 3 of 4 animals local CBF was markedly increased in the absence of macroscopically visible signs of such dysfunction

DISCUSSION

The present results have confirmed our previous observation that intravenous infusion of adrenaline in a dose of 8 μg kg min leads to a pronounced increase in CBF (Berntman et al 1978) This result and that showing an accompanying increase in CMR_{O_2} corroborate previous observations in man (King et al 1952) although the circulatory and metabolic effects induced by adrenaline were far more pronounced in the rat However it is difficult to compare doses in the two studies. The present results have also shown that although adrenaline increases mean arterial pressure, the marked rise in local CBF was present at a time when pressure was again close to normal values. Furthermore although local CBF rose in all brain structures studied some animals showed signs of a marked inhomogeneity For practical reasons Evans blue-albumin extravasation was only estimated macroscopically As the brains under the present circumstances could not be perfused and the concentration of Evans blue was high in the blood, the conditions were not optimal for detecting low grade of tracer extravasation Moreover when the more diffusable BBB tracer sodium fluorescein is given together with Evans blue in acute hypertension the fluorescent areas in the brain are more extensive than the blue areas and Evans blue-albumin extravasation is not observed in all fluorescent areas (Johansson 1974*b*) Thus it seems not unlikely that the BBB was disturbed also in areas with macroscopically apparently intact BBB to protein tracers. The very high CBF in the cerebellum is interesting in light of a recent study in which the BBB dysfunction in adrenaline-induced acute hypertension was quantified with [125]IHSA (human serum albumin) Although multifocal protein leakage was noted in all parts of the brain the highest concentration of the tracer was found in the cerebellum (Johansson 1978) In contrast acute hypertension induced by angiotensin or metaraminol results in extravasation mainly in cortical structures In an accompanying study (Dahlgren et al 1979) it could be shown that when blood pressure was kept constant during infusion of adrenaline in a dose of 8 μg kg min neither CBF nor CMR_{O_2} rose The combined results strongly indicate that the initial increase in blood pressure allows adrenaline to pass the blood-brain barrier and that the enhancement of oxygen consumption and blood flow is caused by the action of the amine on intracerebral adrenoreceptors. We tentatively conclude that the increase in pressure leads to an enhanced translocation of adrenaline across the blood-brain barrier e g. by vesicular transport even when any concomitant translocation of Evans blue protein complex is macroscopically undetectable As shown in another study (Dahlgren et al 1979) the infusion of adrenaline is accompanied by a rise in blood glucose concentration to about 150% of control Since such relatively moderate changes in glucose concentration have not previously been found to influence CBF or CMR_{O} we tentatively conclude that peripheral ef

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nhibition of fast axonal transport and microtubule olymerization in vitro by colchicine and colchiceine

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EDSTRÖM, A HANSON M. WALLIN M. & CEDERHOLM, B Inhibition of fast axonal transport and microtubule polymerization in vitro by colchicine and colchiceine. Acta Physiol Scand 1979 107: 233–237 Received 23 March 1979 ISSN 0001-6772. Department of Zoophysiology University of Lund, Sweden.

The effects of colchicine and colchiceine on fast axonal transport in frog sciatic nerves were studied in vitro. Colchiceine inhibited the transport to about the same extent as colchicine. Preincubation at low temperature potentiated the inhibitory effect of either drug. The polymerization of purified brain tubulin was inhibited by colchiceine at 5–10 times higher concentrations than colchicine The similarity of the effects obtained with colchicine and colchiceine indicates that both drugs arrest axonal transport by interfering with microtubule function Colchicine and colchiceine did not affect the levels of high energy phosphates (ATP and CrP) in frog nerves indicating that reduced energy supply was not responsible for the arrested transport.

Key ords Axonal transport, microtubules, colchicine colchiceine, energy supply

Microtubules (MT) are supposed to play a role in axonal transport (for recent reviews see Dustin 1978, Hanson & Edstrom 1978a). This idea is based mainly on the transport inhibiting effects of antimitotic drugs. No direct proofs exist and contradicting results with mitosis inhibitors have been reported (Byer 1974 Schönbarting et al 1977)

Colchicine blocks axonal transport and inhibits polymerization of tubulin in cell-free systems (Weisenberg & Timasheff 1970 Banks & Till 1975) Differences in degree and time-course of inhibition of axonal transport could be ascribed to its slow penetration into myelinated fibers (Fink et al 1973 Paulson & McClure 1974). Colchicine is presumed to bind only to tubulin and not to intact MT thereby shifting the equilibrium towards tubulin (Wilson & Meza 1973) This process is accelerated by cold and the inhibitory effect of colchicine on axonal transport is potentiated by preincubation at low temperature (Hanson & Edström 1978b Ochs 1976). On the other hand arrested transport has been observed without visible ultrastructural changes (Dustin et al 1975 Norström et al. 1971 Samson 1971), indicating that subtler effects can be sufficient.

The role of MT is central for the understanding of axonal transport and other intracellular motile processes. Colchicine derivatives which retain some of the properties of colchicine while lacking others are therefore useful tools in studies of axonal transport mechanisms The possibility of transport inhibition by colchicine due to effects on cellular processes unrelated to MT has been discussed. Such effects were considered less likely since lumicolchicine, which does not bind to tubulin, lacked transport effects (Banks & Till 1975 Dahlström et al 1975 Paulson & McClure 1974 Price 1974). To obtain further information about this problem another derivative, colchiceine was recently used. It was found to inhibit fast as well as slow axonal transport in the fish optic system (Schönbarting *et al.* 1977) in spite of its inability to bind to rat brain tubulin. Neither did the authors observe ultrastructural changes in colchiceine-treated nerves. In contrast, results in this report raise the possibility that transport inhibitory effects by colchiceine are due to interference with MT and closely similar to those of colchicine.

Axonal transport is closely dependent on energy through ATP (Ochs & Hollingsworth 1971). Col-

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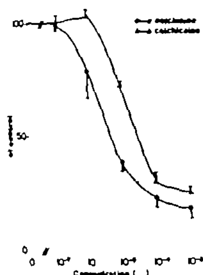
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...e 1 Effects of colchicine and colchiceine on the ...lymerization of MT in vitro Protein samples were ...incubated for 5 min at 4°C with or without drugs (10⁻ ... 10^{-4} M) and the polymerization was measured at 37°C ...ring an experimental period of 25 min. The results are ...sented as per cent of maximum control levels (mean ...lues of 3 expts)

...ESULTS

...lchiceine at 0 1 mM and 0 025 mM reduced the ...cumulation of labelled material in front of a liga-...ure to 47 % and 87 % respectively of control. The ...rresponding values for colchicine were 54 % and ...9% (Table 1). Colchiceine and colchicine thus ...howed about the same inhibitory effect on axonal ...ransport. Neither drug affected protein synthesis in ...he ganglia. Preincubation at 1°C for 3 h in the ...resence of 0.1 mM colchicine or colchiceine re-...duced the accumulated activity to a third of that in ...nerves treated with drug but without cold (data not ...shown). Colchicine was slightly more effective than ...colchiceine under these circumstances.

The levels of ATP and CrP in control nerves after ...cubation for 17 h were 0.35±0.05 and 1 49±0.10 ...mol per mg wet weight (mean ± S E. N =4) re-...spectively These values are only slightly lower ...than in freshly excised nerves Paired nerves incu-...bated in the presence of 0 1 mM concentrations of ...colchiceine or colchicine showed unchanged levels of high energy phosphates.

The effects of colchicine and colchiceine on ...polymerization of calf brain MT are presented in Fig. 1 Both mitosis-inhibitors were found to inhibit the polymerization at high concentrations (10^{-4} and 10^{-5}) and to nearly the same extent. Colchicine was shown to be a more potent inhibitor than colchiceine since it affected the polymerization process even at as low concentrations as 10^{-6} M At this concentration no effect was resolved in the presence of colchiceine

The activities of calf brain MT associated Mg^{2+} and Ca^{2+} ATPases did not deviate from normal values, 2.9 and 3 1 nmol Pi liberated/mg/min at pH 6.8 (Wallin et al 1979) at colchiceine concentrations ranging from 10^{-4} to 10^{-} M.

DISCUSSION

Colchiceine is a metabolite of colchicine in some animals (Schönharting et al. 1974). It is less toxic than colchicine but its effects in the organism are similar e.g. cell division urate induced edema and ^{131}I release from the thyroid are all affected to some extent (Zweig & Chignell 1973 Fitzgerald et al. 1971).

In the optic system of fish colchiceine was reported to be a more effecti e inhibitor of axonal transport than colchicine (Schönharting et al. 1977). In contrast the two drugs had almost equal effects on transport in the frog sciatic nerve in the present study The two agents differ in some properties. Colchiceine is more lipophilic than colchicine and has an affinity for SH-groups (Schnell et al. 1976). It also inhibits phosphatases, probably by interaction with divalent cations (Siebert et al. 1975) Its affinity for tubulin is much lower than colchicine. Schönharting et al. (1977) could not detect any binding of [H]-colchiceine to tubulin but to several other components in a rat brain supernatant. However it has earlier been shown to inhibit [H]-colchicine binding to tubulin to some extent (Zweig & Chignell 1973) Another criterion for MT affinity is its effects on tubulin polymerization. Colchiceine was shown, in the present investigation to inhibit MT assembly at concentrations five to ten times higher than colchicine.

The differences in transport inhibitory effects obtained with the two drugs in the optic and sciatic system might depend on permeability factors and be related to the lipophilic characters of the substances. We have previously shown that several drugs with weak antimitotic activity reduce axonal

Table 1 *Mean values ±S E of radioactivity in ganglia and 4 mm segment of nerve proximal to a ligature 30 mm from the ganglia*

From each animal one nerve was used as a control The ganglion compartments contained 20 μCi [³H]-leucine ml Ringer Colchiceine or colchicine was added to the ganglionic compartments of the experimental nerves and Ringer to all other compartments. *P* values were obtained by use of a two-tailed *t* test for paired preparations

	Conc mM	Counts/min ganglia ×10³	% of control	Counts/min at ligature ×10³	% of control	*P*	No. of animals
Colchiceine Control	0 1	1 793±140 1 861±120	96.4	113±18 241±29	46.8	<0.005	10
Colchiceine Control	0 025	1 330± 48 1 364± 54	97.5	13±21 [illegible]45±23	87 1	<0 02	4
Colchicine Control	0 1	1 961±324 2 044±342	96 0	104±23 195±69	53.5	<0.01	4
Colchicine Control	0 025	1 312±161 1 165±180	112.6	144±[illegible]0 160±27	90.2	n.s.	6

chicine has been shown not to affect energy-dependent neuronal processes other than axonal transport (protein synthesis electrical activity) except at very high concentration However no direct measurements of high energy phosphates (ATP and CrP) have been made in the presence of colchicine or colchiceine and the evidence for an unaffected energy metabolism is indirect In the present study we have measured the effects of transport inhibitory concentrations on the levels of ATP and CrP

MATERIALS AND METHODS

Frogs (Rana temporaria) were used in this study A preparation consisting of the 8th and 9th dorsal ganglia together with the sciatic nerve was dissected out. A ligature was placed on the nerve 30 mm from the ganglia. The two preparations from the same frog were used for experiment and control respectively and were incubated in an apparatus where the ganglia could be separated from the nerve by silicone grease barriers (Edström & Mattsson 1972). Frog Ringer solution with L-[4,5-³H]leucine (105 Ci/mmole 1 mCi/ml The Radiochemical Ctr Amersham) and colchiceine or colchicine were added to the ganglionic compartments of the experimental nerve and ordinary Ringer to the other compartments After incubation for 17 h at 18°C the nerves were cut into 4 mm piece and treated with 5% trichloroacetic acid at 80°C for 1 h After washing in water the ganglia and the nerve piec were solubilized in Soluene 350 (Packard Co) The protein incorporated radioactivity was assessed with a Packard 3375 liquid scintillation spectrophotometer in a 0.55% Permabland III solution (Packard Co) in toluene

ATP and CrP were assayed according to Lowry & Passonneau (1972) Nerves were incubated for 17 h at 18°C in 0 1 mM colchiceine or colchicine solution in Ringer Subsequently the nerves were weighed, dropped into liquid nitrogen and crushed with a pre-cooled steel pestle. ATP and CrP were extracted with 1 M perchloric acid, the supernatant neutralized and the formation of NADPH from glucose and ATP by hexokinase and glucose-6-P dehydrogenase assayed by spectrophotofluorimetry CrP was subsequently converted to Cr and ATP by creatine kinase and the assay repeated

Calf brain tubulin was purified by two cycles of polymerization–depolymerization as described by Borisy et al (1974) and modified by Larsson et al. (1976). One part of brain cortex was homogenized with 1.5 parts ( ) PEG-buffer (PIPES 100 mM EGTA 1 mM GTP 1 mM pH adjusted to 6.8 with NaOH) The homogenate was centrifuged at 25 000 *g* for 1 h at 0–4°C. Tubulin in the supernatant was polymerized at 37°C for 15 min and centrifuged Pellets were stored at −40°C Prior to experiments the pellets were resuspended in PEG-buffer to the desired protein concentration (2.8–3 1 mg/ml) as determined by the method of Lowry et al. (1951) using bovine serum albumen as standard The tubulin sample was preincubated for 5 min at 4°C with or without drugs (10⁻ to 10⁻ M) The time-course of polymerization at 37°C was followed by viscometry in the presence of various concentrations of colchicine or colchiceine

ATPase activity was assayed in samples of MT preparations by measuring liberated inorganic phosphate according to Weil-Malherbe & Green (1951) and modified by Ledig et al. (1975)

Colchiceine (C=O¹⁰-demethylcolchicine) was prepared from colchicine by acid hydrolysis (Boyland & Mawson 1938) The resulting precipitate was washed with ether dissolved in alcohol crystallized from water-alcohol and dried

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transport at mM concentrations in a way which is related to their amphiphilic character (Edström & Hanson 1975) Direct comparisons between the concentrations used in the MT polymerization and transport experiments are probably not possible The sources of nervous tissue and the incubation times differ considerably The present results suggest that the transport inhibitory action by colchiceine is on MT or associated proteins of importance for the polymerization process

Very little is known about the exact mechanisms by which colchicine blocks axonal transport In most cases there is a decreased number of microtubules after colchicine administration (Banks & Till 1975 Hokfelt & Dahlström 1971 Fink et al 1973) However in some nerves transport can be blocked by colchicine without visible structural changes In rabbit optic nerves Karlsson et al (1971) found a normal number of MT in spite of transport block after colchicine administration Identical findings have also been made in the hypothalamo-pituitary system (Dustin et al 1975) Low doses of colchicine might therefore affect the function of MT without degrading them It is possible that the primary transport inhibitory action of both colchicine and colchiceine is on the function of some MT associated protein important for both axonal transport and MT stability

Neither colchicine nor colchiceine affected the ATP or CrP levels of frog nerves Since colchiceine in contrast to colchicine is known to inhibit phosphatases (Siebert et al 1975) its interference with ATPases necessary for the utilization of ATP for axonal transport cannot be excluded Very little is known about transport ATPases in nerves but a possible candidate is brain dynein an MT associated ATPase which however was unaffected by colchiceine

This work was supported by grants from The Swedish Natural Science Research Council (grant no 2535-015) M Bergvalls Stiftelse and Kungliga Fysiografiska Sallskapet Lund. The skilful technical assistance of Inger Antonsson is gratefully acknowledged.

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Effects of cholera toxin on villous tissue osmolality and fluid and electrolyte transport in the small intestine of the cat

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HALLBÄCK, D.-A., JODAL, M. & LUNDGREN, O. Effects of cholera toxin on villous tissue osmolality and fluid and electrolyte transport in the small intestine of the cat. Acta Physiol Scand 1979, 107:239–249. Received 4 Apr. 1979. ISSN 0001-6772. Department of Physiology, University of Göteborg, Sweden.

The effects of cholera toxin on tissue osmolality and on net transport rates of water, sodium, chloride and potassium as well as on unidirectional fluxes of water and sodium were studied in vivo. In all experiments the toxin caused a net secretion of water, sodium, chloride and potassium. The unidirectional sodium transport from tissue to lumen was increased while the flux in the opposite direction was reduced 180 min after cholera toxin instillation. Cholera toxin produced only a small reduction in the villous tissue hyperosmolality created by the intestinal countercurrent exchanger. This reduction was far too small to explain the observed net secretion of fluid and solutes induced by the cholera toxin. Other mechanisms underlying the cholera secretion are discussed.

In reports from this laboratory experimental evidence has been presented for the existence of a countercurrent multiplier in the villi of the feline small intestine (Haljamäe et al. 1973, Jodal et al. 1978). This mechanism establishes the hyperosmolar tissue compartment which, according to Curran & McIntosh (1962), is a prerequisite to explain intestinal water absorption in the absence of, or against a lumen to plasma osmolality gradient. In the study by Hallbäck et al. (1979) it was demonstrated that intestinal absorption changed into secretion when the osmolar gradient along the intestinal villi was abolished by substituting sodium ions in the luminal perfusate with choline.

A pronounced fluid loss from the small intestine can also be produced by exposing the intestinal mucosa to various bacterial toxins. The most thoroughly investigated among such agents is the cholera toxin. The present study was performed to elucidate to what extent cholera toxin influences the osmolar gradient normally present along the intestinal villi during fluid absorption. Fluid and electrolyte transport across the intestinal epithelium was also followed in the experiments.

METHODS

A. *Operative procedures*

The experiments were performed on cats anesthetized i.v. with chloralose (50 mg/kg b.w.) after induction with ether. The animals had been deprived of food for 24 h and had no obvious signs of intestinal infection. The operative procedures and the recordings of blood flow were similar to those described in the paper by Jodal et al. (1975), which should be consulted for details. Briefly, total venous outflow from two sympathetically denervated intestinal segments (mid-jejunum and mid-ileum) was recorded by a drop recorder unit operating an ordinate writer. Mean arterial pressure was measured from the left femoral artery by a pressure transducer (Statham P23AC). Cholinergic influences were eliminated by atropine administration 1 mg/kg b.w.

B. *Recording of intestinal net water transport and experimental procedures*

Net water absorption or secretion were determined according to the method described by Jodal et al. (1975). In short, a recirculating system was coupled to the lumen of each intestinal segment. The volume change of this system was continuously recorded with a volume transducer connected to the system via a Y-tube. Provided no motility occurred the recorded changes of volume reflected net water absorption or secretion. The intraluminal pressure at the outflow end of the segment was kept at about 1

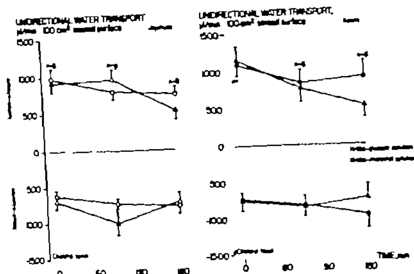


Fig 2 Unidirectional water transport rates in jejunum and ileum from lumen to tissue (upper panel) and from tissue to lumen (lower panel) before and following 30 min incubation with cholera toxin. Bars denote ± S.E.

...ther (water) or μmol/min 100 cm² serosal surface ...lectrolytes).

Cryoscopic technique

General procedure The cryoscopic method including calibration procedures has been presented in detail by ...ald et al. (1978). Briefly the frozen intestinal segment was cut obliquely in cryostat kept at −25°C so as to ...ve 15 μm thick crossections at different levels of the ...ll. The tissue sections were embedded in kerosene between glass plates and transferred to temperature controlled unit consisting of an aluminium block mounted on ... Zeiss standard microscope connected with an automatic camera (Robot Star 50). The temperature of the aluminium block could be set at any temperature between −4.5 and +0.5°C. Thawing of the intestinal tissue was achieved by stepwise increases of tissue temperature which was done automatically with 5 min equilibration period between each step. At the end of each period the tissue section was photographed.

2. *Determination of tissue osmolality* The temperature, at which the last ice crystal thawed in villous crossection, was taken as its melting point. The tissue melting point determined in this study was considered to be measure of the villous core osmolality. The magnification used (about 50 times) was too low to allow any observation of intracellular ice crystals in the villous tissue. The tha ing of the intestinal tissue always started at the villous tips and spred gradually downward until finally the crypt region, the submucosa and the muscularis melted at largely the same temperature. The percentage of melted villous tissue for each temperature level was determined from the photographs. The data were fed into a computer which calculated the tissue osmolality at 5 10, 20 30, 40, 50, 60 70 80, 90% of villous length as well as the mean osmolality between 5 and 30, 5 and 50 and 5 and 100% villous length as described in more detail by Jodal et al. (1978).

E. *Solutions*

The lumen of the intestinal segments was perfused with modified Krebs-Henseleit solution containing (mmol/l): 147 Na 4.7 K 1.2 Mg^{++} 2.5 Ca^{++} 132 Cl^- 25 HCO_3^- 1.2 PO_4^{--} and either 30 glucose or 30 mannitol. The osmolality of the solution was 312±10 mOsm kgH_2O i.e. isotonic with cat plasma.

F. *Statistics*

Statistical significance was tested using the sign test or Wilcoxon nonparametric tests (Siegel 1956).

...er flux from tissue to lumen
...min 100 cm²)

...trol	Cholera
...±76 (4)	774±108 (8)
...96 (5)	717±122 (9)
...±98 (4)	947±172 (4)
...132 (4)	716±185 (7)

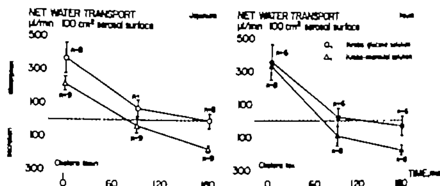


Fig 1 Net water transport in jejunum and ileum before and following a 30 min exposure to cholera toxin Bars denote ±S E

cmH_2O and the intestinal segment was perfused at a constant rate of approximately 1 ml/min by means of a roller pump (Model mp-4 Ismatec Sa, Zürich Switzerland) The temperature of the perfusate entering the segment was continuously monitored with a thermocouple thermometer (Electrolab Copenhagen) and kept at about 38°C by a heating pad Recirculation was prevented by including a large reservoir (700–1 000 ml) in the circulating system

After a control period of 45 min samples of the perfusing medium entering and leaving each segment were collected Thereafter 200–300 mg of crude cholera toxin (freeze dried culture filtrate NIH lot 001) per 10 cm intestine dissolved in 5–6 ml bodywarm physiologic saline solution was placed in the lumen of each segment Both ends of the segments were closed and the toxin was exposed to the mucosa for 30 min. The toxin was then gently washed out with 30–50 ml warm perfusing medium and the perfusion was again started Samples of the perfusate entering and leaving the segments were taken at 90 and 180 min after the start of cholera toxin exposure Immediately after the collection of the last samples from the perfusion system the segments were rapidly extirpated and momentarily frozen in isopentan precooled to about −150°C in liquid nitrogen.

C *Biochemical and radioactive measurements*

In order to calculate water and electrolyte fluxes across the intestinal epithelium samples (2–3 ml) were taken from the perfusates entering and leaving the intestinal segments The samples were collected in plastic tubes The sodium and potassium concentrations in the samples were determined with a flame photometer (Eppendorf) and the chloride concentrations were determined with a chloride meter (Corning Eel 920 Chloride Meter Halstead Essex England).

For determination of water and sodium unidirectional transport from lumen to tissue 62.5 µCi HTO and 5 µCi ^{22}Na (0 05 µg) were added per liter of the perfusion fluid Radioactivity was measured by a liquid scintillator (Packard Tri-carb liquid scintillation spectrometer model 3320 Quenching was corrected for by using an external γ standard The coefficient of variation of the radioactive measurements was below 1% All analyses were carried out in duplicates

Intestinal net transport of sodium, potassium and chloride as well as unidirectional transport of water and sodium were calculated as described by Hallbäck et al (1979) The net transport and the fluxes were related to serosal area and expressed as µl/min 100 cm² serosal

Table 1 *Rates of net water transport and unidirectional water fluxes before and 180 min after a 30 min intestinal incubation with cholera toxin*

The intestinal segments were perfused with a Krebs-glucose or a Krebs-mannitol solution Mean values ±S E. An asterisk denotes statistical significance

	Net water movement (µl/min×100 cm²)		Water flux from lumen to tissue (µl/min×100 cm²)	
	Control	Cholera	Control	Cholera
Jejunum				
Krebs-glucose	370±90 (8)	−1±44 (8)	997±139 (8)	773±110 (8)
Krebs-mannitol	15±4 (9)	−174±70 (9)	928±170 (9)	542±118 (9)
Ileum				
Krebs-glucose	356±106 (6)	−3±58 (6)	1 091±145 (6)	915±18 (6)
Krebs-mannitol	330±87 (8)	−179±30 (8)	1 15±184 (6)	579±157 (7)*

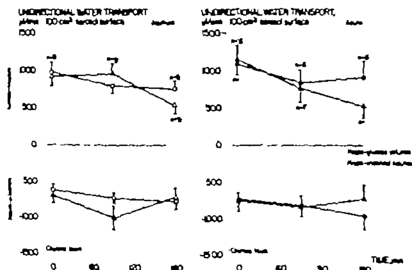


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F Statistics

Statistical significance was tested using the sign test or Wilcoxon nonparametric tests (Siegel 1956).

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…trol	Cholera
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…±96 (9)	774 108 (8)
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…±132 (4)	947±172 (4)
	716 185 (7)

…[illegible] (7)

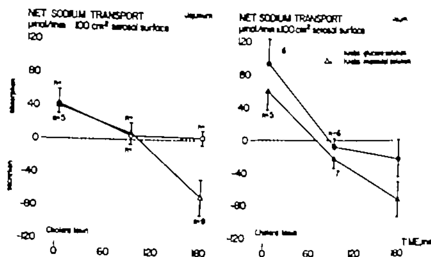


Fig 3 Net sodium transport in the small intestine before and following a 30 min exposure to cholera toxin Bars denote ± S E

RESULTS

Water transport Fig. 1 illustrates the net water transport rate in the jejunum (left panel) and in the ileum (right panel) before 90 and 180 min after cholera toxin exposure Table 1 summarizes net and unidirectional water transport in the jejunum and the ileum during control and after 180 min cholera toxin incubation A positive value denotes a net water transport from lumen to tissue (absorption) and a negative value a net transport in the opposite direction (secretion) Statistically significant differences are indicated in the Table

It is evident from Table 1 and Fig 1 that cholera toxin caused a marked and statistically significant reduction in net water transport particularly when the intestinal segments were perfused with a Krebs-mannitol solution Fig shows the unidirectional water transport rates in the jejunum (l panel) and in the ileum (right panel) plotted a Fig 1 The observed reductions in net water tra port rates in the jejunum and the ileum (Fig 1) se to be due largely to a reduction in the water tra port rates from lumen to tissue (Table 1).

Sodium transport Fig. 3 summarizes the re on net sodium transport rates plotted in a sim way as for net water transport (Fig. 1). Table presents net and unidirectional sodium fluxes du ing control and after 180 min cholera toxin exp sure Statistical significance is indicated in the Table

Both in the jejunum and in the ileum cholera to led to statistically significant decline in net sod absorption In fact in many experiments a net s cretion of sodium was recorded Sodium secreti

Table ? *Rates of net sodium transport and unidirectional sodium fluxes before and 180 min after a intestinal incubation with cholera toxin*

The intestinal segments were perfused with a Krebs-glucose or a Krebs-mannitol solution Mean values ± SE asterisk denotes statistical significance

	Net sodium movement (mmol/min × 100 cm²)		Sodium flux from lumen to tissue (mmol/min 100 cm²)	
	Control	Cholera	Control	Cholera
Jejunum				
Krebs-glucose	41 9±17 1 (7)	9_8 9 (7)	81 1+17 0 (6)	61.5 11 7 ( )*
Krebs-mannitol	39.3±9.. (5)	69 9_ 1 6 (9)	100 +14 (5)	.5 7 8 5 ( )*
Ileum				
Krebs-glucose	93 4±79 1 (6)	.. 5±.. 0 (6)	119 8 .3 3 (5)	50 7 17 5 (6
Krebs-mannitol	60..±...6 (5)	7? 5+ 1 3 (7)	107 5 13 7 (4)	34 7 9 8 ( )

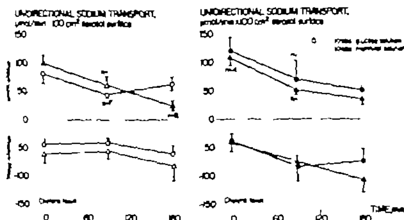


Fig 4 Unidirectional sodium transport rates in jejunum and ileum from lumen to tissue (upper panels) and from tissue to lumen (lower panels) before and after a 30 min exposure to cholera toxin. Bars denote ± S E.

as particularly prominent when the perfusate con ained mannitol but not glucose The study of uni- irectional fluxes (Table 2 and F g 4) revealed that he decrease of net sodium absorption could be scribed to both a reduction of the lumen to tissue odium flux and an augmentation of tissue to lumen lux.

Chloride transport Fig 5 illustrates net chloride ransport before and after exposing intestinal seg ments to cholera toxin Table 3 summarizes the esults obtained before and 180 min after cholera oxin incubation

It is clear that cholera t xin caused significant eductions in net chloride transport n both the ejunum and ileum particularly when using Krebs-mannitol solution With no glucose in the perfusate a marked net loss into lumen of chloride ions was observed

Potassium transport The results on potassium transport are presented in Fig. 6 and Table 3 In both intestinal segments small rate of potassium absorption during the control period was changed into a small secretion of potassium 180 min after the start of the cholera toxin exposure

Tissue osmolality F g. 7 illustrates the osmotic gradient along the illus in ntestinal segments ex turpated 180 min after the beginning of the cholera toxin incubation Results obtained with glucose containing solutions are indicated with circles while those obtained with Krebs-mannitol solutions are denoted with triangles in the jejunum (left panel) and in the ileum (right panel) Tip and mean osmolality in the upper 5 to 30 5 to 50 and 5 to 100% of illous length are given in Table 4 In this table is also indicated osmolality data obtained during control i previous nvestigation (Hallback et al 1979) Exposure to cholera toxin decreased the arious villous tissue osmolalities measured in this study The reduction was, however statistically significant only in the upper parts of segments exposed to Krebs-glucose solution, and at the illous tip of a jejunal segment perfused with a Krebs-mannitol solution.

Blood flow Total blood flow n the two intestinal segments including their lymph nodes during control conditions and after cholera toxin incubation are hown in Table 3 Cholera toxin caused statistically significant increases in total blood flow in all

um flux from tissue to lumen
ol min 100 cm²)

trol	Cholera
17 8 (6)	
14 7 (5)	60 6 ± 15 4 (7)
	81 5 76 2 (8)
L I (5)	
16 2 (4)	73 1 0 (6)
	107 3 ± 21 6 (7)*

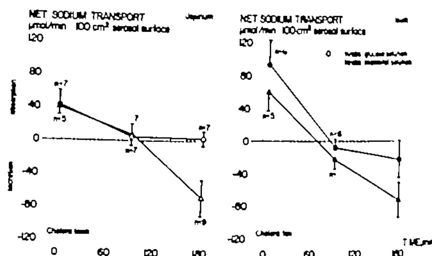


Fig 3 Net sodium transport in the mall intestine before and following a 30 min exposure to cholera toxin Bars denote ± S E

RESULTS

Water transport Fig. 1 illustrates the net water transport rate in the jejunum (left panel) and in the ileum (right panel) before 90 and 180 min after cholera toxin exposure Table 1 summarizes net and unidirectional water transport in the jejunum and the ileum during control and after 180 min chol era toxin incubation A positive value denotes a net water transport from lumen to tissue (absorption) and a negative value a net transport in the opposite direction (secretion) Statistically significant differ ences are indicated in the Table

It is evident from Table 1 and Fig. 1 that cholera toxin caused a marked and statistically significant reduction in net water transport particularly when the intestinal segments were perfused with a Krebs mannitol solution Fig. shows the unidirec tional water transport rates in the jejunum (left panel) and in the ileum (right panel), plotted as in Fig. 1 The observed reductions in net water transport rates in the jejunum and the ileum (Fig. 1) seem to be due largely to a reduction in the water transport rates from lumen to tissue (Table 1)

Sodium transport Fig 3 summarizes the results on net sodium transport rates plotted in a similar way as for net water transport (Fig. 1). Table presents net and unidirectional sodium fluxes during control and after 180 min cholera toxin exposure Statistical significance is indicated in the Table

Both in the jejunum and in the ileum cholera toxin led to statistically significant decline in net sodium absorption In fact in many experiments a net secretion of sodium was recorded Sodium secretion

Table *Rates of net sodium transport and unidirectional sodium fluxes before and 180 min after intestinal incubation with cholera toxin*

The intestinal segment were perfused with a Krebs-glucose or Krebs-mannitol solution Mean values S E asterisk denotes statistical significance

	Net sodium movement (mmol/min 100 cm²)		Sodium flux from lumen to tissue (mmol/min 100 cm²)	
	Control	Cholera	Control	Cholera
Jejunum				
Krebs-glucose	41 9_17 1 (7)	9±8 9(7)	81 1 17 0 (6)	63 5 11 7 (1)*
Krebs-mannitol	39.3±9 (5)	69 9_ 1 6 (9)	100 ±14 (5)	_5 7±8 5 (8)*
Ileum				
Krebs-glucose	93 4±79 1 (6)	[illegible] 0 (6)	119 8 _3 3 (5)	50 7 17.5 (6)*
Krebs-mannitol	60 [illegible] 6 (5)	-77 5± 1.3 (7)	107 5+13 7 (4)	34 7 9 8 (7)

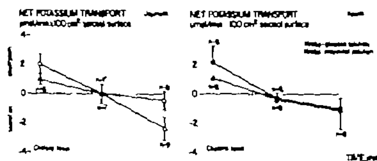


Fig. 4. Net potassium transport in jejunum and ileum befor and following a 30 min exposure to cholera toxin. Bars denote ± S E.

he majority of studies in vitro as well as in vivo gue that glucose absorption and glucose associated sodium transport are unaltered by cholera exin exposure (Serebro et al 1968 Iber et al 1969 Field 1971). On the other hand it has been suggested that cholera toxin abolishes the electrolyte transport in the neutral NaCl carrier (for references, see Field 1974).

The reduction of the unidirectional sodium transport rate from lumen to tissue after cholera toxin exposure, seen in the present study (Fig. 4) supports the view that cholera toxin caused a depression of solute uptake in agreement with some earlier reports (Phillips 1968, Swallow et al. 1968 de Jonge 1975 Kreja et al 1978) The transport mechanism affected by the toxin is however difficult to ascertain from our black box results, but they are consistent with the view expressed above that the cholera toxin mainly decreased the NaCl-coupled absorption at the luminal surface

The view that cholera toxin initiates or augments an active unidirectional solute secretion from tissue to lumen can be traced back to Snow (1855) and Cohnheim (1890) Based on physiologic studies at least two secretory mechanisms in the jejunum and ileum have been proposed, i.e an active transport of bicarbonate and by some authors, of sodium chloride The effect of cholera toxin on these transport processes is not quite clear According to Powell (1974) the rate of transport of both HCO_3^- and NaCl are increased, while Field (1971) favours the view that cholera toxin initiates a chloride secretion not present during physiologic circumstances. An increased outflux of sodium was observed in this study in agreement with earlier reports (Banwell et al. 1968 and 1972, Phillips 1968 Swallow et al 1968 Iber et al 1969 Love 1969 and 1971 Grayer et al. 1970, Love et al. 1972, Kreja et al 1978 Wald et al 1977) However the present data do not allow any conclusions to be drawn with regard to the cellular actions involved in the observed increase of tissue to lumen flux of sodium

In our experiments no consistent differences between the jejunal and ileal responses to cholera toxin exposure could be demonstrated, as far as net or unidirectional fluid and solute secretion rates are concerned. This is at variance with the observations by Carpenter et al (1968) who reported that the cholera secretion in vivo in the jejunum was rich in chloride ions while that in the ileum had a high concentration of bicarbonate ions. The latter solute was not measured in the present study but no difference in net chloride transport was observed between jejunum and ileum (Fig. 5).

In the present experiments the intestinal segments were perfused with solutions containing either mannitol or glucose Adding glucose to the

...al blood flow ...min × 100 g tissue)	
...ntrol	Cholera
14 (8)	
4 (9)	41 5 (8)*
	36±4 (9)*
3 (6)	
4 (8)	38±6 (6)
	33±3 (8)*

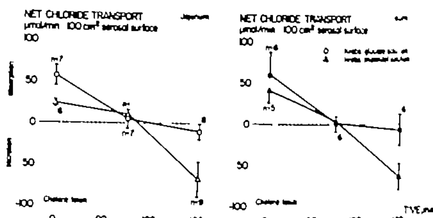


Fig 5 Net chloride transport in the small intestine before and following a 30 min incubation with cholera toxin Bars denote ± S E

intestinal segments except in the ileum during Krebs-glucose perfusion

DISCUSSION

The present study has shown that exposing the cat small intestine to cholera toxin in vivo turned a net fluid absorption into a net secretion within 1 to 2 h as earlier reported from this laboratory (Cedgård et al 1978) Concomitant with this fluid loss a net secretion of Na Cl and K into the lumen was observed as also reported in a large number of in vivo studies on other species including man (Benyajati 1966 Banwell et al 1970) dog (Carpenter et al 1968 Swallow et al 1968 Moore et al 1971 Krejs et al 1978) and rabbit (Leitch et al 1966 Love 1969 Norris et al 1969)

It is generally agreed that water transport across the intestinal epithelium is a passive process secondary to the active transport of electrolytes, mainly sodium chloride In accordance with this view cholera net secretion of fluid and electrolytes may be produced in 3 ways (1) By a decrease of the normally occurring salt flux from lumen to tissue (2) by an increase in oppositely directed transport or (3) by a combination of 1 and 2. During physiological circumstances at least 3 major transport mechanisms are responsible for the active transport of sodium across the intestinal epithelium (Schultz et al 1974) A rheogenic glucose-facilitated transport system and a chloride-coupled electrically neutral transport mechanism are located at the apical membrane of the enterocyte while a Na-K dependent ATPase carrier is situated at the basolateral membrane The lastmentioned transport system is generally believed not to be influenced by the cholera toxin (Schultz et al 1974). Furthermore

Table 3 *Net chloride transport rate net potassium transport rate and total intestinal blood flow before and 180 min after a 30 min intestinal incubation with cholera toxin*

The intestinal segments were perfused with Krebs-glucose or Krebs-mannitol solution Mean values ± S E asterisk denotes statistical significance

	Net chloride movement (µmol/min 100 cm²)		Net potassium movement (µmol/min 100 cm²)	
	Control	Cholera	Control	Cholera
Jejunum				
Krebs-glucose	57±1 (7)	10±9 (8)	01 0 66 (8)	0 46 0 6 (8)
Krebs-mannitol	4±4 (6)	68± 1 (9)	0 97 0 36 (6)	37±0 78 (9)*
Ileum				
Krebs-glucose	60±27 (6)	7 19 (6)	13 1 10 (6)	0 99±0 69 (6)*
Krebs-mannitol	41±15 (5)	65±16 (8)	1 08 0 19 (8)	−1 16 1 4 (8)

perosmolality especially at the villous tip. This perosmolality has been proposed to be the main ng force for passive water and solute absorp- n in vivo (Haljamae et al. 1973; Jodal 1973; Jodal al. 1978; Hallbäck et al. 1978) and in a recent per by Hallbäck et al. (1979) a correlation be- een tissue hyperosmolality and net water uptake s demonstrated. One major aim of the present udy as to elucidate to what extent cholera toxin fluences the villous hyperosmolality created by e countercurrent multiplier. The results demon- rate that cholera toxin caused only moderate duction of illous tissue hyperosmolality (Fig. 7 nd Table 4), hich can be ascribed to at least two ossible mechanisms.

First, an intestinal hyperemia occurred after holera toxin exposure mainly localized to the ucosal part of the intestinal wall, where blood low doubled as compared to control (Cedgård et al. 978). This observation probably implies that also llous blood flow is increased during cholera and if o, the efficiency of the countercurrent exchanger ecomes decreased (Lundgren 1967; Winne 1975). uch an effect of vasodilatation was demonstrated y Jodal et al. (1978) showing that illous tissue smolality was lowered to approximately the same evel as observed in the present study when pro- ounced intestinal hyperemia was induced (blood flow 150 ml/min 100 g). The fact that intestinal blood flow during cholera toxin exposure was in- creased only to 40–60 ml/min 100 g suggests that the "flow factor" is of fairly subordinate importance in explaining the decrease of villous tissue os- molality.

Second, the multiplying effect of the intestinal countercurrent exchanger is influenced by the rate of net solute transport across the illous epithelium. It was, for example demonstrated by Haljamäe et al. (1973) that the administration of ouabain mark edly reduced the sodium gradient along the villous tip in all probability reflecting a decreased effi- ciency of the countercurrent multiplier. In analogy with this, t seems reasonable to assume that the decreased tissue osmolality along the villi during cholera toxin exposure observed in the present study was mainly due to depressed transport capacity of sodium across the villous enterocytes as discussed in detail above.

The reduction of villous hyperosmolality induced by cholera toxin incubation in the small bowel was far too small to account for the observed secretion rate of fluid and solutes (Hallbäck et al. 1979). In the normal small intestine a net loss of fluid occurs when the mean tissue osmolality in the upper third of the villus was below 400 mOsm/kg H_2O (Hallbäck et al. 1979) while in the present study secretion was apparent already when tissue osmolality in the same villous parts was around 600 mOsm/kg H_2O. Part of this difference may be explained by a decrease in epithelial hydraulic conductivity demonstrated in experiments in vitro during cholera toxin exposure (Nellans et al. 1974; Powell 1974), lowering lumen to tissue flux of water particularly in the segments perfused with a Krebs-mannitol solution (Table 1). However the most likely explanation seems to be an increased fluid and solute transport from tissue to lumen, as discussed above. Such an increase in water transport into the lumen was not seen in the present study. This difficulty in finding a correla- tion between the sodium and water transport rates from tissue to lumen was also seen in other studies that measured unidirectional water fluxes in vivo during cholera (Swallow et al. 1968; Banwell et al. 1972). The explanation may be found in the meas- urements of unidirectional fluxes in vivo as men- tioned above and possibly also in the fairly short observation time of the present study (cf. Krejs et al. 1978).

The tissue osmolality recorded at the villous tip in the present study is equivalent to a driving force for water around 5 000 mmHg (667 kPa) assuming a reflection coefficient of 0.6 for sodium chloride (Fordtran et al. 1965). With such forces involved it seems less likely that the villi represent the main site for electrolyte and fluid secretion in cholera.

100% of
... length
(mOsm/kg H_2O)

...ntrol	Cholera
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... 19 (4)	459 ± 14 (8)

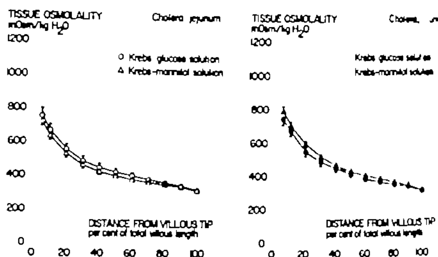


Fig 7 Tissue osmolality in the villi of the jejunum (left panel) and ileum (right panel) 180 min after a 30 min incubation with cholera toxin Data were obtained from intestinal segments either perfused with Krebs-glucose solutions (circles) or with Krebs-mannitol solution (triangles) Bars denote ± S E At many point of the Figs, the S E is so small that it falls within the area of the ymbols For number of observations see Table 4

perfusate markedly reduced the net secretion rates of fluid and electrolytes caused by cholera toxin exposure Our data thus support findings by Carpenter et al (1968) Serebro et al (1968) and Iber et al (1968) indicating that glucose facilitated electrolyte absorption was unaffected by the chol era toxin Glucose has proved to be most useful in the treatment of Asian cholera (Phillips 1964 Lancet 1975)

The effects of cholera toxin on the cellular mechanisms of electrolyte transport have been studied mostly in in vitro preparations which offer the possibility for more detailed cellular studies than in vivo investigations (cf Schultz et al 1974)

The difficulty of standardizing in vivo studies is reflected in the present study by the comparatively large scatter of results An even more striking example of this is the fact that the literature contains reports which demonstrate that the lumen to tissue flux of sodium is decreased (Swallow et al 1968 de Jonge 1975 Krejs et al 1978), unaltered (Iber et al 1969 Love 1969) or increased (Hamed et al 197? Love et al 197? Wald et al 1977) after exposing the small intestine to cholera toxin

In numerous reports from this laboratory it has been demonstrated that the vascular arrangement of the small intestinal villus can function as a countercurrent multiplier and thereby create a marked

Table 4 *Tip and mean osmolality in various portions of intestinal villi 180 min after a 30 min exposure to cholera toxin*

Control values were recorded in a previous study (Hallback et al 1979) The intestinal segment were perfused Krebs-glucose or with a Krebs mannitol solution Mean values ± S E ( ) An asterisk denotes statistical significance

	Villous tip osmolality (mOsm/kg H_2O)		5 to 30% of villous length (mOsm/kg H_2O)		5 to 50% of villous length (mOsm/kg H_2O)	
	Control	Chole	Control	Cholera	Control	Choler
Jejunum						
Krebs-glucose	1 0_ ±30 (9)	64_68 (6)	689 3 (9)	598 55 (6)	590_3 (9)	53] 48 (6)
Krebs-mannitol	901±50 (8)	751 30 (7)	65 34 (8)	588±3 (7)	580 28 (8)	5_3 50 (7)
Ileum						
Krebs-glucose	940±33 (8)	74 _63 (6)	679 _ (8)	584±54 (6)	591±19 (8)	557 47 (6)
Krebs-mannitol	895±57 (8)	789 44 (8)	680 43 (8)	6_4 4 (8)	601_34 (8)	553 19 (8)

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Thus the present study supports the theory of an intestinal secretion mechanism initiated by cholera toxin the site being anatomically separated from the absorptive area of the villus and probably located to the crypt regions in the small intestine (Hendrix 1971 1972) The evidence for and against such an intestinal secretion in the crypts obtained in in vitro and in vivo expts. was recently reviewed by Hendrix & Paulk (1977) A further observation that may be of importance in this connection is the nervous mechanism proposed by Cassuto et al (1979) to be involved in the pathogenesis of cholera.

The cholera toxin was kindly supplied to us by Dr Jan Holmgren Department of Medical Microbiology University of Göteborg The present research was supported by grants from the Swedish Medical Research Council (14X -855), from the Swedish Society for Medical Sciences, from Harald and Greta Jeansson s Fund from Magnus Bergvall's Stiftelse and from the Faculty of Medicine, University of Goteborg

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KREJS G J BARKLEY R M READ, N W &

ggression-provoked renin release from extrarenal d extrasubmaxillary sources in mice

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BING J & POULSEN K Aggression-provoked renin release from extrarenal and extrasubmaxillary sources in mice. Acta Physiol Scand 1979 107 251–256. Received 9 April 1979 ISSN 0001-6772. University Institute for Experimental Medicine, and Institute for Biochemistry Royal Dental College, Copenhagen, Denmark.

In submaxillary sialoadenectomized and nephrectomized mice aggressive beha iour provoked 5 to 40-fold increases in plasma renin concentration. The changes in renin concentration with time were different in different groups of confronted mice with only partial correlation between the pattern and the observable degree of fight. The changes were similar in sialoadenectomized mice with untouched kidneys as in sialoadenectomized and nephrectomized, indicating that aggression causes no measurable, if any renal renin release Repeated aggression with 2 hourly intervals provoked repeated renin relase from extrarenal and extrasubmaxillary sources. The renin concentrations of different organs showed the same mutual relationship as in other mammals, but were about 10-fold higher Splenectomy was without effect on the aggression-provoked renin release Antibodies against pure mouse renin neutralized the renin in plasma and organs, which contained only insignificant, if any pepsin activatable inactive renin. Adrenaline, apomorphine, carbachol and dihydralazine were as isoprenaline and noradrenaline without effect on renin release in sialoadenectomized and nephrectomized mice

Key ords Splenectomy hypotension, neurotransmitters, renin-aggression.

n mice aggressi e behaviour provokes vast increase in plasma renin concentration, which eaches about 100 to 600-fold the normal in normal nd in nephrectomized mice Smaller but still marked increases are found in submaxillary sialoadenectomized mice (Bing & Poulsen 1979*b*). Thus, most of the increase in plasma renin found after aggression in normal and nephrectomized mice is due to release of submaxillary renin In sialoadenectomized mice the renin release was thought to originate from the kidneys, but an extrarenal release could not be excluded The aim of the present investigation was to study if aggression provokes an increase in plasma renin concentration in mice hich ha e been sialoadenectomized and nephrectomized, and if so if this increase can account for the renin release in sialoadenectomized mice ith untouched kidneys. Further studies aimed at lucidating differences in the effect of ar ious degrees of aggression the origin of the released renin and the mechanism of renin release

MATERIAL AND METHODS

Animals, male albino mice of the Danish State Serum Institute strain most of which eighed 40–65 g, but some weighing 25 to 40 g, were individually housed for at least 30 days. Submaxillary sialoadenectomy was performed at least 10 day and nephrectomy 2 h before the start of the experiments. In some mice the spleen was removed together with the kidneys. The operations were performed during short term ether anesthesia after pretreatment with penicillin. No mouse was used for more than one experiment

Aggression was provoked by grouping for 20 min of 3 to 4 previously isolated mice in small cage as described by Valzelli (1969). In most cases aggression was provoked and intensified by addition of one or two previously isolated non-operated mice.

Blood was sampled from tail ein during light ether anesthesia. Tissues ere removed after decapitation in order to drain as much blood as possible. Extraction of the tissue samples after addition of 1% Triton 100 and determination of *renin concentration* in plasma and extracts as performed as described by Poulsen & Jørgensen (1974), using their trapping radioimmunoassay for angiotensin I. Extracts of mesentery- striated muscle- and lung tissue were performed after pooling of pieces from 2

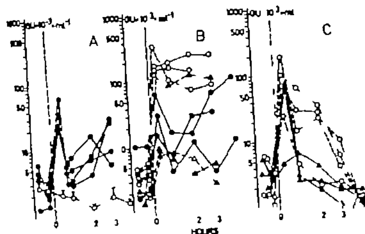


Fig. 1. (A) The time course of the plasma renin concentrations in 4 sialoadenectomized and nephrectomized mice in which grouping caused vigorous fighting (marked ●), and the mean values and SEM of that of 9 mice which were kept isolated (♂). (B) Values from three groups of mice (marked ○, ● and △) which fought after addition of non-operated mice to intensify fighting. (C) Values from 2 groups of mice (marked ○ and ▲) which after grouping only reacted with starts. In B and C the individuals of different groups are marked by different symbols. The ordinates indicate the concentration in G.U. 10^{-3} ml^{-1} on a logarithmic scale. The abscissae show the time in hours before and after the start of confrontation, which is marked by a dashed line.

hours after the start of the aggression, including 4 livers, 4 spleens and 3 hearts, containing 68 to 95, 72–90 and about 5 G.U. ×10⁻ per g, respectively.

Pepsin activation of inactive renin in plasma and organ extracts from sialoadenectomized and nephrectomized mice

The procedure for pepsin activation showed that plasma and extracts contained little if any inactive renin. It was performed on 11 plasma samples. Six of these were taken 2 (=3) or 4 (=3) hours after nephrectomy of previously sialoadenectomized mice, which were kept isolated without any confrontation. Their renin concentration was 0.6 to 2.5 G.U. 10 ml before the pepsin treatment, which resulted in only small increases, less than 13 in 3 samples and 19, 31 and 67% in the 3 other samples. Five plasma samples obtained from similarly pretreated mice 20 to 60 min after the start of an aggressive fighting of 20 min duration contained from 4.8 to 160 G.U. 10 ml. In all of these the pepsin activation was without significant effect. After pepsin treatment of extracts of 5 spleens, 4 livers and 1 heart, containing 8 to 120 G.U ×10 ×g, 2 extracts showed small (20 and 47%) increase, while the renin concentration was less than 15% increased in 2 and quite unchanged in the other 6 samples.

VI. Renin concentration and content in some organs of sialoadenectomized and nephrectomized mice

The mean renin concentration in extracts of 28 livers removed from 0 to 8 hours after nephrectomy was 75 G.U. ×10⁻ ×g (range 30–130), the values in this and the following groups not changing significantly with time during the 8 hours. There was no significant difference between the concentration in livers taken from the group of mice in which aggression had been provoked (=15) and those taken from the group in which the mice had not been confronted (=13), the mean values being 82 and 67, respectively. In 19 extracts of livers removed 4 hours after nephrectomy the mean renin concentration was significantly lower, being about 15 G.U. ×10⁻ per g (range 0.3–36) in both aggressive and non-aggressive mice (p=0.001). As a 50 g mouse has a liver weight of about g, the total renin content in the liver is about 150 G.U. ×10⁻⁶ during the first 8 hours and about 3 G.U. ×10⁻ 4 hours after binephrectomy.

to 4 mice Antibody against pure submaxillary mouse renin prepared as described by Malling & Poulsen (1977) was used for antirenin neutralisation of enzymatic activity of some of the samples which for this purpose were mixed with antibody at 7 4 *Pepsin activation* was performed as previously described (Nielsen Malling & Poulsen 1978)

The effect of different *neurotransmitters* and other pharmacological agents on renin release in sialoadenectomized and nephrectomized mice was determined using conscious mice In these as in all other experiments. In cases where the substances were given by intra-arterial injection a catheter was placed in the femoral artery at least 2 hours before the experiments ( ) Blood pressure determination was in some experiments performed using a Tybjærg Hansen transducer and a Servogor 511 recorder The agents used were adrenaline given as a 1 min lasting intraarterial infusion of 1 μg/g b w ($n=7$) after pretreatment with 0 5 μg/g dihydralazine The dopaminergic agonist apomorphine was given subcutaneously in doses of 0 1 or 0 3 mg/kg ($n=5$) or 1 to 5 mg/kg ($n=4$) of apomorphine chloride Carbachol was given subcutaneously in doses of 3 ($n=4$) or 12 ($n=3$) μg/kg and in 3 mice in doses from 200 to 800 μg/kg. The use of the agents was based on the well documented effect of some neutrotransmitters and of hyptension on renal and (or) submaxillary renin release (see Bing & Poulsen 1979*a*) Significance of changes in renin concentration were estimated by Student's *t* test

RESULTS

I *Change in plasma renin concentration with time after different degrees of aggression of sialoadenectomized and nephrectomized mice*

In 4 mice which fought vigorously and looked exhausted after the ?0 min lasting fight the plasma renin concentration rose markedly at the end of the fight The control values from 9 sialoadenectomized and nephrectomized mice which were kept isolated during the experiment are marked ♂ (Fig 1 A) To 3 groups each of 3 sialoadenectomized and nephrectomized mice 2 non-operated mice were added in order to initiate and intensify the aggression The results are seen in Fig 1 B The results from groups of 3 and 4 mice respectively in which the confrontation did not result in any fight but only in nosing and small starts with small sudden movements are seen in Fig 1 C Independent of the degree of fighting a marked increase in renin was seen immediately after the confrontation ($p<0\ 01$ $n=?0$) The renin concentration varied considerably in the following hours but stayed elevated for several hours in most animals. Only in the 7 animals (Fig 1 C) involved in the lowest degree of reaction the renin concentration had reached basal values at the end of the experiments.

II *Comparison between the effect of aggressive behaviour on plasma renin concentration in sialoadenectomized mice which were either nephrectomized or sham operated before the confrontation*

Nine isolated previously sialoadenectomized mice were divided into 3 groups In ? of these ? mice were nephrectomized and 1 sham-operated, and in the third group 1 was nephrectomized and ? sham-operated Two hours after the operations blood was taken for renin assay whereafter the mice belonging to a group were confronted provoking aggressive behaviour After the end of the ?0 min fight the changes in plasma renin concentration were followed for about 6 h As shown in Fig. ? there was no significant difference between the mean values of the nephrectomized (○) and the sham-operated (●) for the first 3 hours after the confrontation, but at the end of the experiments the values of the sham-operated were somewhat higher (p 0 C than those of the nephrectomized

III *Effect on the plasma renin concentrations of 3 consecutive confrontations causing aggressive behaviour with about 2 hourly intervals*

The effect of repeated fightings was studied in 3 sialoadenectomized mice with untouched kidneys (not shown) and in 3 previously sialoadenectomized mice which had been nephrectomized h before the first fighting (Fig 3) The three consecutive fightings resulted in a 3-fold (range ?0), 60-fold (range 50) and 14-fold (range 40) increase in renin concentration respectively ($n=6$)

IV *Antirenin neutralization of renin in plasma and organs from sialoadenectomized and nephrectomized mice*

In 8 plasma samples taken at the end of a 20 min lasting aggression ($n=6$ with a renin concentration from 63 to 195 G U ×10 ×ml) or 4 and 6 hours later ($n=$ containing about 30 G U ×10 ml) addition of antirenin completely neutralized the enzymatic effect of the renin A total neutralization was also obtained in 11 extracts of organs removed

III. Effect of adrenaline, apomorphine, carbachol and dihydralazine on the renin in sialoadenectomized and nephrectomized mice

In mice, which after previous sialoadenectomy had been nephrectomized about 2 h in advance, adrenaline, the dopaminergic agonist apomorphine and carbachol did not provoke any change in plasma renin concentration. The doses, way of administration and number of mice are given in Material and Method. Also injection of 0.5 µg/g dihydralazine, which in 5 mice provoked a prolonged decrease in blood pressure from a mean of 130 mmHg (range 120–140) to a mean of 60 mmHg (range 40–75) was without effect on the plasma renin concentration.

DISCUSSION

In previously submaxillary sialoadenectomized and about 2 hours nephrectomized mice aggression was found to provoke a marked increase in plasma renin concentration. The concentration rose from 2–7 G U×10⁻⁴ ml⁻¹ to values about 5 to 40-fold higher. The pattern was different in different groups without an evident relation to the observable degree of fighting (Fig. 1 A and B). There was a similarly marked increase in plasma renin concentration in mice, which after grouping did not fight but only reacted with starts. In these mice the plasma renin returned faster to normal values (Fig. 1C).

In sialoadenectomized and nephrectomized mice aggression provoked similar changes in renin concentration as previously found in sialoadenectomized mice with untouched kidney (Bing & Poulsen 1979*b*). A direct comparison of the values in groups of these two types of differently pretreated mice showed that the changes with time were not significantly different during the first 3 hours after the confrontation. The values were, however, somewhat higher in the mice with untouched kidneys 6 hours after the confrontation. This difference can be due to release of renal renin caused by the repeated bleedings, the total loss of blood being about 400 µl.

When sialoadenectomized and nephrectomized mice were fighting repeatedly each new fight provoked a new increase in plasma renin concentration, which in this way could reach higher values than after a single fight (Fig. 3). But even after single fight the increase is unexpectedly high, when compared with the total renin content in the organs studied. The liver and spleen, which had the highest concentrations, had a total content of about 160 G U ×10⁻⁴, and the concentrations of heart, lung and mesentery were much lower. The relation between the concentration in the different organs was about the same as previously found in other mammals (Eskildsen 1973, Ganten et al. 1977), but both in the organs and in plasma the concentration was about 10-fold higher in mice than in the other animals. Both in plasma and in organ extracts the renin was totally neutralized with antibodies against pure submaxillary renin. The marked renin relase can probably not be due to transformation of inactive renin, as both plasma and organ extracts contained only small, if any, pepsin activatable renin.

The source of the renin released in sialoadenectomized and nephrectomized mice is unknown. The pronounced decrease in the renin content of the liver about 24 hours after the nephrectomy was found in non-aggressive as well as in mice which had been aggressive. And the unchanged renin concentration in the liver during the first hours after the aggression makes it improbable that the liver is the main source. As splenectomized and sham-splenectomized mice reacted in the same way, the spleen cannot play a major role as source. The mesentery vessels were in a previous study found to release renin when dogs were subjected to hypotension (Ganten et al. 1970). This was, however, not confirmed in experiments on rats (Wernze & Sekl 1972) and dogs (Fagard et al. 1974), and the present study shows that in mice dihydralazine provoked marked hypotension without causing any increase in plasma renin. So far the source of the renin released by aggression in sialoadenectomized and nephrectomized mice has not been determined. The evidence for persistent vascular renin activity in nephrectomized rat (Swales & Thurston 1973, 1977) makes it possible that the vessels found throughout the body are the source from where the renin is released.

It is unknown how the aggressive behaviour provokes the renin release. In the previous experiment (1) isoprenaline and noradrenaline were found to be unable to provoke renin release in sialoadenectomized and nephrectomized mice. The present study shows the same lack of effect after injection of adrenaline, the dopaminergic apomorphine and carbachol.

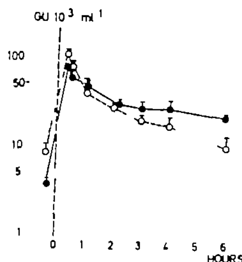


Fig The mean values of aggression-provoked changes in plasma renin concentration with time in 4 sialoadenectomized mice with untouched kidneys (marked ●) and 5 sialoadenectomized and nephrectomized mice (O) Vertical lines indicate SEM Ordinate and abscissa as in Fig. 1

In extracts of spleens removed from 0 to 8 hours after nephrectomy the renin concentration was not significantly lower in 13 isolated mice than that of the 15 mice in which aggression had been provoked with mean values 127 (range 40–400) and 218 (range 25–500) G U ×10⁻ per g respectively In 19 spleens removed 4 h after nephrectomy the renin concentration was the same as in those removed after 0–8 h in 9 mice which were kept isolated the concentration being 176 G U ×10 per g (range 30–220) However the mean concentration of renin in spleens removed 24 h after nephrectomy in 10 mice in which aggression had been provoked was markedly lower than in spleens removed 0–8 h after nephrectomy the mean value being 77 (range 10–180) ($p<0.05$) The mean total renin content of the spleen of a 50 g isolated mouse was about 10 G U × 10⁻⁴

In the other organs studied the renin concentrations were markedly lower than in the liver and spleen In the heart the concentration was about 3 G U ×10 ×g both in the first 8 h ($n=9$) after nephrectomy and after 24 h ($n=1$) in mice which had not been confronted After aggression the values were similar 8 h ($n=1$) and 4 h after binephrectomy ($n=9$) The mean total renin content of a heart was 0 6 G U ×10 In mesentery striated muscle- and lung tissue of 16 mice in which no aggression had been provoked the mean renin concentrations in G U ×10 per g were 1 7 (range 0 9–2.7) 0 6 (range 0.3–1 4) and 1 8 (range 0 8–3 6) respectively with no difference between the concentration in tissue taken 0 to 8 and 4 h after nephrectomy

VIII *Effect of splenectomy on the aggression-provoked increase in plasma renin concentration*

In 3 groups of each 2 splenectomized and sham-splenectomized mice all of which were sialoadenectomized and nephrectomized a 20 min aggression was provoked 2 h after the operation Immediately after the fight the renin concentration was unusually high being 960 G U ×10 ×ml in one of the splenectomized mice In the other splenectomized mice the mean concentration was 165 G U ×10 ×ml⁻¹ (range 42–420) which was the same as in the sham-splenectomized in which the mean was 165 (range 42–405) Also in the following 4 hours the values of the splenectomized and sham-operated followed each other In one group of 2+2 mice the renin concentration stayed high as in the group marked 0 in Fig 1B the mean concentrations being 168 and 163 milli G U per ml 4 h after the confrontation Of the other 4+4 mice 1 sham-operated died after about 1½ h In the other 7 mice the renin concentrations were falling throughout the experiments the mean value 4 h after the confrontation being 22 G U ×10⁻ ×ml (range 8–38) in the splenectomized and 17 (range 6–35) milli G U per ml in the sham-splenectomized

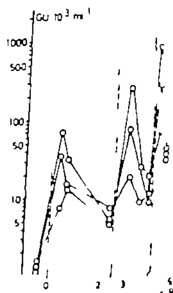


Fig 3 The time course of the plasma renin concentration in 3 sialoadenectomized and nephrectomized mice in which aggression was provoked 3 times marked by dashed vertical lines Ordinate and abscissa as in Fig 1

ta Physiol Scand 1979 107 257–261

hanges in lipoprotein-lipase activity and lipid stores ı human skeletal muscle with prolonged heavy exercise

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LITHELL, H ORLANDER, J SCHÉLE, R., SJÖDIN B. & KARLSSON J Changes in lipoprotein lipase activity and lipid stores in human skeletal muscle with prolonged heavy exercise Acta Physiol Scand 1979 107 257–261 Received 19 April 1979 ISSN 0001-6772. Department of Geriatrics, University of Uppsala, the Department of Animal Nutrition, Swedish University of Agricultural Sciences, Uppsala, and the Laboratory for Human Performance (FOA 57), Stockholm Sweden.

Lipoprotein-lipase (LPL) activity and intracellularly stored triglycerides were determined in muscle biopsies taken before and after an 85 km skiing race from 7 volunteers. The triglyceride stores were larger in slow twitch than fast twitch fibres (proportions 5 1 before the race) The LPL activity increased and the triglyceride stores in slow twitch fibres decreased during the race The best trained subjects had the largest TG stores before the ace and their TG stores also decreased most during the race. These subjects also had ery small increases of LPL activity The least trained subject on the other hand showed a 6-fold increase of LPL activity The high post-race LPL activity in less trained subjects indicates higher capacity for uptake of fatty acids from serum TG as compared to the mor trained subjects

Key ords Intermyofibrillar fat, lipoprotein-lipase activity muscle fibre types, prolonged exercise

uring normal dietary conditions the relative exer se intensity (i e actual $\dot{V}_{O_2}$/maximal $\dot{V}_{O_2}$) de rmines to what xtent energy is supplied from arbohydrates or fat (Christensen & Hansen 939a) Christensen & Hansen (1939b) found that ncreasingly more energy wa derived from fat ombustion during prolonged ex rcise at a relative ntensity of about 65% but no fat adaptation was een at intensities abov 75% (Hermansen et al 1967). Fat combustion during exercise was higher fter a fat-rich diet than after a carbohydrate-rich iet (Christensen & Hansen 1939) or normal diet Bergstrom et al 1967) During exercise the uptake of free fatty acids (FFA) from plasma into muscle s related to it concentration in the plasma (for ref see Gollnick 1977) The increase in fat combustion during prolonged exercise however can only part ly be xplained by an increased uptake of plasma free fatty acids (for ef see Froberg 1977) Fatty acids might also be supplied from plasma triglycerides (TG) which ar hydrolysed by the enzyme lipoprotein-l pase (LPL) localized at the endothelium in the muscle capillaries (Robinson 1970) A work-induced increase of the enzyme ac ti ity should mean that an increased amount of fatty acids were made available to the muscles (Robinson 1970) In addition an increased utilization of intramuscular TG stores that are hydrolyzed by the intracellularly located so called hormone-sensitive l pase (Bjorntorp & Furman 1962) has been demonstrated both in animals (Baldwin et al. 1973 Reitman et al 1973) and humans (Howald 1975 Essén et al 1977)

The objecti e of the present investigation has been to evaluate the changes in both LPL activity and intracellular lipid stores in muscle tissue d ring extremely prolonged, heavy exercise

SUBJECTS AND METHODS

Seven male physical education students who participated in an 85 km cross-country skiing ace (the Vasa ace 1977)

CONCLUSION

Aggression provokes a marked release of renin in sialoadenectomized and nephrectomized mice The source of this extrasubmaxillary and extrarenal renin could not be located to any single organ and is possibly located to vessels throughout the body A similar renin release was not provoked by injection of several catecholamines or carbachol and was not provoked by prolonged marked hypotension Aggression causes only insignificant if any release of renal renin in sialoadenectomized mice

This study was supported by grants from King Christian X Foundation and the Foundation of Insurance Companies of 1952 The excellent technical assistance of Vibeke Meyland-Smith is gratefully acknowledged

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Table 2. Skeletal muscle lipoprotein-lipase (LPL) activity and volume fraction of subcellular components in slow twitch (ST) and fast twitch (FT) muscle fibres before and after the race

Mean values ± S.D. Ranges are given within parentheses. n=7

	Before	After	Change	*P*
LPL (mmol × min⁻¹ g⁻¹)	18.4±7.0 (11.9–31.4)	58.9±33.2 (16.1–116.5)	+40.5±32.2 (1.2–100.7)	<0.02
Lipid droplets				
ST (%)	0.95±0.32	0.35±0.39	−0.60±0.30	<0.01
FT (%)	0.19±0.15	0.12±0.10	−0.07±0.24	>0.40
P_{dif} ST–FT	<0.001	<0.10	<0.02	
Sarcoplasm				
ST (%)	10.4±1.7	5.5±2.6	−4.9±2.7	<0.01
FT (%)	8.9±1.1	9.6±3.1	0.7±3.5	>0.66
P_{dif} ST–FT	<0.05	<0.05	<0.01	
Mitochondria				
ST (%)	4.80±0.75	5.86±1.43	+1.06±1.34	<0.10
FT (%)	2.74±0.23	2.77±0.83	+0.03±0.84	>0.90
P_{dif} ST–FT	<0.001	<0.001	<0.10	
Myofibrils				
ST (%)	83.9±2.0	88.3±2.2	+4.4±2.9	<0.01
FT (%)	88.1±1.4	87.5±2.8	−0.6±3.6	>0.60
P_{dif} ST–FT	<0.001	>0.40	<0.01	

...ce and that the increase was negatively correlated to the amount of ski-training before the race. As the amount of ski-training was closely correlated to both performance time ($r=-0.86$, $P<0.05$) and $\dot{V}_{O_2}$ max ($r=0.73$, $P<0.10$) it is not possible to state which of the three factors—if any—that was the primary determinant for how much the LPL activity increased.

No significant relation was seen between LPL activity and the percentage of ST fibres. This could be due to the small sample size but the finding is supported by a later study with a larger sample (Lithell et al. to be published). In rats, however, red muscles have 10-fold higher LPL activity than white muscles (Linder et al. 1976, Borensztajn et al. 1975).

Before the race more lipid was stored in ST than in FT fibres and well-trained subjects had on an average larger lipid stores. This finding is in accordance with the observations of Morgan et al. (1969) and Hoppeler et al. (1973) of 2.5 times higher lipid content in trained than in untrained human muscles.

During the race lipid stores decreased significantly only in ST fibres. Well-trained subjects used on an average more of the total stores (= ST + FT) than the less trained subjects did.

Others have reported decreased content of TG in human muscle after prolonged heavy exercise, i.e. after a previous Vasa race (Froberg & Mossfeldt 1971) and after a 100 km race (Howald 1975) but not for ST and FT fibres separately. However Reitman et al. (1973) found that muscle TG decreased by 70% in red, 25% in intermediate and not at all in the white portion of rats quadriceps muscles. This is in agreement with Paul's statement (1975) that intramuscular (and even more so extramuscular) fat depots of trained animals and subjects supply more fatty acids as energy substrate during exercise of long as well as of short duration as compared to the fat depots of untrained animals.

The decrease in the volume fraction of sarcoplasm in ST fibres indicates a dehydration of this fibre type. This dehydration is probably connected with the glycogen depletion which Forsberg et al. (1978) found to be more frequent in ST fibres (85%) than in FT fibres (5%) after a previous Vasa race as water is released when glycogen is oxidized (Sréter 1963, Kozlowski & Saltin 1964).

There was a relationship between the change in LPL activity and the amount of lipid droplets before the race, so that the well-trained subjects who had the largest amount of lipid droplets had the smallest increase of LPL activity. There might be several reasons for this. One possibility is that in the well-trained subjects fatty acids released from the in-

Table 1 *Characteristics of the 7 subjects*

	Mean	S D	Range
Age (yrs)	26 7	3 ?	23 1–32
Height (cm)	175 7	4.3	169 7–180 8
Weight (kg)	76 4	6.8	66.6–83 1
$\dot V_{O_2}$ max (l × min)	4 76	0.37	4.16–5
$\dot V_{O_2}$ max (ml × kg × min)	6? 7	6 6	57.3–78.0
Muscle composition (%ST)	46 4	14 6	21–64
Amount of ski training (km)	429	291	100–1 000
Performance time (h)	8.07	1.39	5.3 –9 6

volunteered for the study The subjects were of normal body weight and height but varied considerably in capacity (within the upper part of the normal range) and training level as *indicated in Table 1 Mean* performance time was 8 07 h (range 5.32–9 6)

Biopsies were taken from the vastus lateralis the day before the race and immediately after the race with a needle technique (Bergstrom 196) The biopsies were divided into three portions One was embedded in a plastic medium for histochemical analysis by staining sections for myosin ATPase and identification of slow twitch (ST) and fast twitch (FT) fibres (Gomori 1941 Padykula & Herman *1955)* The second portion was used for electron microscopy Small pieces of muscle tissue were fixed in 4% phosphate-buffered glutaraldehyde *pH 7 4 (at least 2 h at* 0°C) washed post-fixed in 1% veronal-buffered osmium tetroxide washed in Tyrode s solution and dehydrated in ethanol After treatment with propylene oxide the muscle pieces were embedded n Epon. Approximately 60–70 nm thick longitudinal sections were cut with a LKB Ultrotome I stained with uranyl acetate and lead citrate and examined in a Siemens Elmiskop 101 About 25 micrographs (final magnification about 25 000) were taken at random from two tissue blocks per biopsy Volume fractions of myofibrils mitochondria lipid droplets and sarcoplasm (including sarcoplasmic reticulum and transverse tubules) were estimated stereologically according to Weibel (1969) The study was restricted to the fibrillar space of the muscle fibres The fibres were classified as ST or FT according to Z-line width (Payne et al 1975 Wroblewski & Jansson 1975 Eisenberg & Kuda 1976 Tomanek 1976, Bylund et al 1977) measured to the nearest 0.1 mm with a measuring magnifier Only clearly demarcated Z-lines were chosen for measurement excluding the fuzzy matrix present at the Z line border

Muscle LPL activity was determined in the third portion of the biopsy as described *by Lithell & Boberg (1978)* The biopsies were incubated in reaction medium containing H trioleate labelled Intralipid (Vitrum, Stockholm Sweden) The release of H-oleic acid was used a measure of LPL activity The activity was expressed as nmol fatty acids (FA) released per minute and per gram wet weight of muscle tissue (nmol FA × g min) This method determines the activity of the heparin-releasable LPL located at the capillary endothelium (Robinson 1970) Specificity tests have excluded that ?? of the determined enzyme activity is due to hormone sensitive lipase.

Maximal oxygen uptake ($\dot V_{O_2}$ max) was determined during treadmill exercise one week before the competition according to the Douglas bag procedure (Saltin & Åstrand 1967)

Statistical methods. Student s *t*-distribution was used when testing the means of individual changes (before-after) and differences (ST FT) and when testing correlation coefficients.

RESULTS

Skeletal muscle LPL activity averaged 18.4 nmol FA × g^{-1} × min^{-1} (range 11 9–31 4) before the race and the average increase during the race was ?? (range 8–637%) (Table ?) The increase in LPL activity was negatively related to $\dot V_{O_2}$ max ($r = -0$ 85 $P<0.05$) amount of ski training before the race ($r = -0$ 9? $P<0.01$) and fitness level i e $\dot V_{O_2}$ max divided by body weight (r 0.7? $P<0$ 05) and positively related to performance time ($r = 0$ 83 $P<0$ 05)

Before the race the volume fraction of lipid droplets was higher in ST than in FT fibres (0.95% ± 0 19% $P<0$ 001) The total volume fraction of lipid droplets (i e in ST *and* FT fibres) before the race was positively correlated to the amount of training ($r = 0$ 90 $P<0$ 05) (The correlations for volume fraction in ST and FT separately $r = 0.46$ and ?? were not statistically significant) During the race the volume fraction of lipid droplets decreased in ST fibres (from 0 95% to 0.35% *P<0 01)* but not significantly in FT fibres (Table 2)

The decrease in volume fraction of lipid droplets in the combined muscle fibre population (ST FT) correlated positively to $\dot V_{O_2}$ max ($r = 0.86$, $P<0.05$) and amount of training ($r = 0.79$ $P<0.05$), i e well trained subjects used more of the intramuscular lipid stores than the less trained subjects did.

A 46% decrease in the volume fraction of sarcoplasm (from 10.4 to 5 5% $P<0$ 01) was found in the ST fibres

A negative relationship ($r = -0.81$ $P<0.05$) was present between the increase in LPL activity and the pre race volume fraction of fat droplets in the muscle fibres (ST + FT)

DISCUSSION

One major finding in this study was a marked increase in skeletal muscle LPL activity during exer

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There was a relationship between the change in LPL activity and the amount of lipid droplets before the race, so that the well-trained subjects who had the largest amount of lipid droplets had the smallest increase of LPL activity. There might be several reasons for this. One possibility is that in the well-trained subjects fatty acids released from the in-

tramuscular lipid droplets might repress the synthesis of LPL (Pykälistö 1970) Another possibility is that there is a time lag from the time when the synthesis rate increases before increased LPL activity can be detected at the endothelial level During feeding experiments it was found that the heparin releasable LPL activity in adipose tissue was not significantly increased until 6 h after the feeding started (Pykälistö et al 1975) Furthermore very heavy work leading to exhaustion after one hour did not significantly affect the LPL activity in muscle tissue in man (Lithell et al 1979) However during heavy work for 8–9 h the LPL activity in muscle increased also in well trained subjects (Cedermark et al to be published)

The results of the present study confirmed earlier findings that the energy supply to the muscles during heavy physical work is partly derived from intracellularly stored lipid and that well-trained individuals both have larger stores and more readily use them However a selective decrease of lipid droplets in human ST fibres has to the best of our knowledge not been reported earlier Hermansen et al (1967) found lower respiratory quotients in trained as compared to untrained subjects indicating a relatively larger fat combustion in trained subjects. Those subjects who performed the race fastest also had the best capability for fat combustion in terms of larger stores of lipid and a greater readiness to use it If the time lag before LPL activity increases at the endothelial level is about 6 h training does not seem to shorten this period but to result in larger lipid stores that can be used before increased amounts of fatty acids derived from serum triglycerides can be available

We are indebted to Miss Kristina Stensjö for skilful technical assistance

The study has been supported by grants from the Swedish Medical Research Council (Project No 4251 and 5446) and the Research Council of the Swedish Sport Federation.

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ta Physiol Scand 1979 107 263–267

he effect of ingestion of amino acids glucose and fat on irculating neurotensin like immunoreactivity (NTLI) in man

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ROSELL, S. & ROKAEUS Å. The effect of ingestion of amino acids, glucose and fat on circulating neurotensin-like immunoreactivity (NTLI) in man. Acta Physiol Scand 1979 107 263–267 Received 19 April 1979 ISSN 0001-6772. Department of Pharmacology Karolinska Institutet, Stockholm, Sweden.

The effect of ingestion of amino acids (Vamin N) glucose, and fat (Intralipid) on the concentration of neurotensin-like immunoreactivity (NTLI) in plasma was determined in 6 healthy male volunteers After ingestion of Intralipid (55 ml, 200 mg ml⁻) there was significant increase in the plasma concentration of NTLI. The calculated integrated total NTLI response (6.7±2.5 nM over 180 min) was statistically significant. Although isocaloric amounts of Vamin and glucose also increased the plasma concentration of NTLI these increases were not statistically significant. The data indicate that fat is an important stimulus for the elease of NTLI from the small intestine. It is suggested that neurotensin, or metabolite in blood may be hormone involved in the postprandial inhibition of gastric motility and gastric acid secretion elicited from the small intestine.

Key ords Enterogastrone, gastrointestinal hormone, neurotensin

In several species, including man, immunocytochemical investigations have shown that neurotensin, a tridecapeptide (Carraway & Leeman 1973 1975), is stored in a specific type of cell (the N cell) hich is present in the mucosa of the ileum and jejunum and, to a lesser extent, in the colon and duodenum (Carraway & Leeman 1976, Orci et al 1976, Sundler et al. 1977 Helmstaedter et al. 1977 Polak et al 1977 Frigerio et al. 1977). In anesthetized dogs higher concentrations of neurotensin-like immunoreactivity (NTLI) were found in blood draining from the ileum than in the arterial blood (Mashford et al. 1978b), which indicates that neurotensin is released into the blood from the ileum It has been shown in man that ingestion of mixed meal causes a marked rise in the concentration of NTLI in blood (Besterman et al. 1978, Mashford et al 1978a) and food appears to exert a major influence on the blood concentration of NTLI (Mashford et al. 1978a). Neurotensin administered by intra enous infusion in low doses in dogs elicits inhibition of gastric motility (Andersson et al. 1977), suppres ion of gastric acid secretion (Andersson et al. 1976), vasodilatation in the gastrointestinal region and a delayed vasoconstriction in subcutaneous adipose tissue (Rosell et al. 1976) Taken together these data suggest that neurotensin, or metabolite thereof in the blood may be a hormone serving some physiological function in relation to the digestion of food (Mashford et al 1978a).

The present experiments were designed to find out if one or more of the principal constituents of a mixed meal has a dominant effect on the venous concentration of NTLI in humans.

METHODS

Venous plasma NTLI was examined in 6 healthy male olunteers, aged 22–47 years after an overnight fast; the experiments being started between 8 and 10 m. On separate days, the subjects were given amino acids (430 ml as Vamin N Vitrum), glucose (30 g as 300 ml Glucose, ACO 100 mg ml⁻) or fat (11 g as 55 ml Intralipid, Vitrum, 200 mg ml⁻) orally in isocaloric amounts (about 460 J 105 kcal). After having ingested Vamin, glucose or Intralipid, sufficient ater was given to make the final volume of fluid administered 500 ml. One subject was given 500 ml of

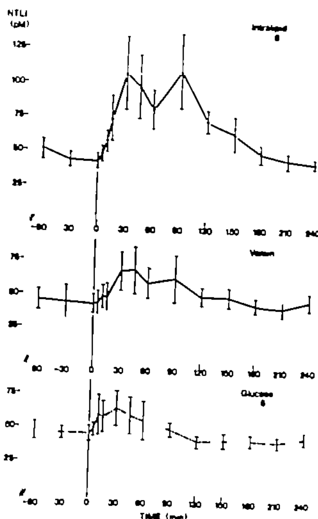


Fig 1 Concentrations of NTLI in plasma after oral administration of Intralipid Vamin or glucose Number of subjects=*n* The vertical bars indicate the standard error of the means. $=p<0.05$ $=p<0.01$

water orally on a separate day Each volunteer stood or sat in an upright position while ingesting the nutrients Heparin (Vitrum 50 unit ×ml blood) and aprotinin (Trasylol 100 K.I U ×ml blood) were added to the venous blood samples The samples were kept in ice until centrifugation at +4°C The plasma was stored at −20°C until subjected to radioimmunoassay

The method used in the radioimmunoassay of neurotensin will be presented in detail elsewhere (to be published) and only a brief description of the method is given here The antiserum (0-7701) was raised in rabbits by injection of neurotensin (Beckman) coupled to bovine serum albumin using 1-ethyl-3 (3-dimethyl-amino-propyl)-carbodiimide HCl (Goodfriend Levine & Fasman 1964). The antigen was injected together with Freund' complete adjuvant at 3–8-week intervals. This antiserum reacts with neurotensin (NT) NT (1 1) (Gln) NT and (Gln) NT (1 11) but not with NT (4–13) or smaller C terminal fragments of NT It shows no cross-reactivity with the gastric inhibitory polypeptide (GIP) the vasoactive intestinal peptide (VIP) secretin, cholecystokinin-33, cholecystokinin-39 pancreatic glucagon, substance P somatostatin bovine pancreatic polypeptide ... bombesin, gastrin-17 gastrin 34 or with trypsinized gastrin 34 which presumably contains the N-terminal heptadecapeptide Synthetic neurotensin (Beckman) was labelled with I 125 using the chloramine T method (Hunter & Greenwood 196_) The assay can measure levels down to 5 pM Work is under way to determine whether ... measuring some metabolite of neurotensin in addition to neurotensin itself Experimental data indicate that ... the actions of neurotensin (vasoconstriction in adipose tissue) may be caused by substance(s) derived from neurotensin (Rosell et al. 1978)

In order to estimate the integrated NTLI response the area under the plasma concentration time curve from the time of ingestion of the nutrients to 180 min thereafter when the NTLI concentration had returned to control, (AUC_{0-180}) was calculated using the trapezoidal rule. The control value was estimated graphically by drawing a straight line between the control sampling points before the ingestion of the nutrients and the sampling points 180–240 min post ingestion. To calculate the integrated response the area under the straight line over 180 min was subtracted from the total AUC_{0-180}.

Statistical evaluation of the data was performed with the Wilcoxon signed rank test and Student's *t*-test for correlated means The results are reported as mean standard error of the mean ($M \pm$ S E)

RESULTS

After an overnight fast the mean concentration of neurotensin like immunoreactivity (NTLI) in plasma was 51±6.7 pM ($n=6$) Following ingestion of Intralipid the concentration of NTLI increased in all the subjects Two peaks of elevated NTLI in plasma were seen one at 30 min and the other at 90 min (109±?7 pM) with return of the plasma level of NTLI to control levels by 180 min (Fig 1). The calculated integrated NTLI response was 6.7 ...

Table 1 *Integrated responses of NTLI after oral administration of nutrients (nM×180 min)*

Subject	Intralipid	Vamin	Glucose
AB	18	6.9	5.3
SR	8 1	0 9	0
AJ	7 6		
ÅR	5	1 4	0
JL	3	0 5	0
GO	1 6		0 3
Mean S E	6.7 5	4 1.5	1 1 1 0
	$p<0.05$	n s.	n s.

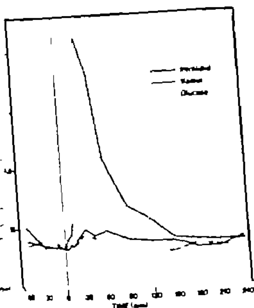


Fig. 2. Concentration of NTLI in plasma in one subject after oral administration of Intralipid, Vamin or glucose.



Fig. 3. Concentration of NTLI in plasma in one subject after oral administration of Intralipid, Vamin or glucose.

d over 180 min (Table 1). After ingestion of Vamin the NTLI in blood rose in all 4 subjects but there was an appreciable increase in only 2 subjects and the mean peak concentration did not differ significantly from the mean resting concentrations. Similarly the calculated integrated NTLI response was not significant (Table 1). After ingestion of glucose the NTLI level in blood increased in only 1 of 5 subjects. The mean concentration of NTLI in blood and the integrated response were not significantly different from resting values (Fig. 1 and Table 1).

Although all subjects responded with an increased concentration of plasma NTLI after ingestion of Intralipid there was a pronounced interindividual variability particularly as regards time–response relationships. In some subjects there was a clearcut increase of NTLI in plasma already at 10 min with a maximum at 30 min (Fig.) whereas in another an increase in plasma concentration was found at 90 min (Fig. 3). These interindividual differences partly explain the finding that the mean plasma concentration curve of NTLI after Intralipid ingestion shows peak values (Fig. 1). However in two subjects the plasma NTLI concentration curves did show two peaks at 30 min and 90 min respectively (Fig. 4). The integrated NTLI response after Intralipid ingestion also showed pronounced interindividual variations, the lowest value being 1.6 nM over 180 min and the highest 18 nM over 180 min. The subject with the most pronounced NTLI response after Intralipid also responded with an increased release of NTLI after ingestion of Vamin, glucose and the same volume of water (500 ml) (Fig. 4).

DISCUSSION

The present results indicate that fat is a strong stimulus for the production of increased levels of

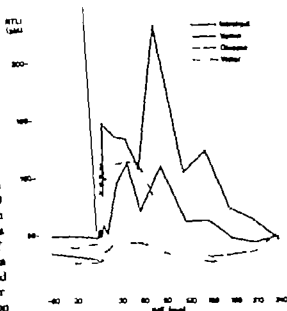


Fig. 4. Concentration of NTLI in plasma in one subject after oral administration of Intralipid, Vamin, glucose or water.

NTLI in blood Although ingestion of amino acids and glucose caused an increase in NTLI in some subjects fat was clearly the most active nutrient in this respect In one subject responding with an increased NTLI to all 3 nutrients 500 ml of water also caused a slight increase of NTLI This may indicate that the distension of parts of the gastrointestinal tract may be of significance for the release of NTLI However distension is not of importance in all individuals since ingestion of 500 ml of Vamin or glucose did not change the concentration of NTLI in blood in all subjects We have reported earlier that there was no response of NTLI to alcohol coffee or heavy exercise (Mashford et al 1978*a*) Our new data further support and extend the hypothesis that food and particularly fat exerts a major effect on the plasma concentration of NTLI in humans

The rise in NTLI is probably caused by release of neurotensin from the glandular neurotensin-containing cells in the small intestine presumably due to a direct exposure of the gastrointestinal tract to nutrients Evidently the fat does not have to be absorbed since intravenous administration of Intralipid in the same dosage as was ingested orally did not influence the concentration of NTLI (to be published) The segment of the gut from which nutrients elicit release of neurotensin is not known One possibility is of course that neurotensin is released from the jejunum-ileum where most of the neurotensin-containing cells are located (cf Bloom & Polak 1978) In some of the subjects an increased NTLI level was noticed 10 min after the ingestion of fat. This may seem a short period for the fluid to pass into the small intestine and there cause a release of neurotensin which is detected as increased NTLI in blood However it has been shown in fasting normal subjects that chyme appears in the small intestine 100 cm distal to the Treitz ligament from 10 to 30 min after administration of a homogenized standard meal (Clain Liang & Malagelada 1978) There is also the possibility that some neurotensin is released from its storage sites by the action of releasing hormones or a nervous reflex originating from the proximal part of the gastrointestinal tract. Further studies are needed to identify the site from which neurotensin is released following food intake and to elucidate the release mechanisms

Since the end of the nineteenth century it has been known that gastric secretion and gastric motility are inhibited when ingested fat reaches the small intestine (Ewald & Boas 1886, Babkin 1950) In man several studies have been made in recent years to elucidate the action of fat in the small intestine on gastric secretion and gastric motility Windsor Cockel & Lee (1969) found that instillation of fat into the duodenum and small intestine depressed a near maximal gastric acid output by approximately 50% Similarly Christiansen and coworkers (1976) have demonstrated a pronounced inhibition of gastric acid secretion in the human small intestine following infusion of Intralipid 40-5 cm distal to the ligament of Treitz. Clain Liang & Malagelada (1978) diverted the chyme from the intestine at a site 100 cm distal to the ligament of Treitz, which resulted in an increased gastric secretory response to a meal They suggested that the distal small intestine plays an inhibitory role in postprandial gastric secretion Thus, several groups have demonstrated in man the existence of a potent postprandial gastric acid inhibitory mechanism in the distal part of the small intestine which is activated by fat Experimental evidence has been obtained that this inhibition elicited by fat (and also by acid and hypertonic glucose Gregory 1967) is caused by a hormone from the intestinal mucosa and Kosaka & Lim (1930) named it enterogastrone Several gastrointestinal hormones have been suggested to have enterogastrone activity (e g. secretin cholecystokinin and gastric inhibitory peptide GIP) although none of these has been established as the enterogastrone (cf Babkin 1950 Gregory 1967 Brown et al 1975 and Cataland 1974) Our finding that ingestion of fat causes a pronounced increase in the plasma concentration of NTLI during 2 h or more suggest that neurotensin or a metabolite thereof may be involved in the postprandial inhibition of the gastric motility and gastric acid secretion elicited from the small intestine The localization of most of the glandular neurotensin-containing cells to the small intestine where fat seems to elicit the inhibition of gastric acid secretion and gastric motility supports such a hypothesis In addition i v infusion of neurotensin in anesthetized dogs in low doses (4-8 pmol×kg ×min) inhibits motor activity in the antral part of the stomach (Andersson et al. 1977) and gastric acid secretion (Andersson et al 1976) However before neurotensin or some metabolite thereof can be ascribed a physiological role as an enterogastrone it remains to be shown that NTLI

ncentrations in blood after ingestion of fat are in e range found after the administration of doses of ageneous neurotensin capable of inhibiting gastric otility or gastric secretion in humans.

his study was supported by the Swedish Medical Research Council (grant no. 3518) and by the Astra Research undation. We thank Drs R. Carraway (Harvard Univ ston, Mass.), R. Chance (Lilly Res. Lab Indianapolis, diana), K. Folkers (Univ of Texas, Austin, Texas) and Mutt (Karolinska Institutet, Stockholm) for the generous supply of various peptides.

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ıcreased hepatic glycogen synthetase and decreased hosphorylase in trained rats

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GALBO H SAUGMANN P & RICHTER, E. A.. Increased hepatic glycogen synthetase and decreased phosphorylase in trained rats. Acta Physiol Scand 1979 107 269–272. Received 23 April 1979 ISSN 0001-6772. Institute of Medical Physiology B University of Copenhagen and Department of Medicine, Marselisborg Hospital, Aarhus, Denmark.

Rats were either physically trained by 12 wk swimming program or were freely eating or weight matched, sedentary controls Trained rats had higher relative liver weight and total hepatic glycogen synthetase (EC 2.4 1 11) activity and lower phosphorylase (EC 2.4.1 1) activity than the other groups of rats. These changes may partly explain the demonstrated training-induced increase in glucose tolerance None of the findings could be ascribed to differences in food intake or body weight.

Key ords. Liver enzymes, glycogen, glucose tolerance test, exercise, metabolism

ıly few studies of the effect of physical training n hepatic metabolic functions have been preented. It has been reported that training increases epatic malic dehydrogenase activity (Sangster & Beaton 1966) as well as the concentrations of the ytochromes and the activities of glycerol-P dehydrogenase and succinate dehydrogenase (Kraus & Kinnen) whereas hepatic glyceride ynthesis does not change (Askew Huston & Dohm 1973). Furthermore the hepatic glycogen concentration has been found to be markedly increased after training (Bald in et al 1975 Galbo et al 1977) A less pronounced training-induced increase in muscle glycogen concentration (Holloszy & Booth 1976, Galbo et al 1977) led to investigation of the effects of training on enzymes invol ed in muscle glycogen ynthesis. An increased total glycogen synthetase activity in skeletal muscles of humans and rodents was found (Holloszy & Booth 1976).

In the present study we ha e measured hepatic glycogen ynthetase and phosphorylase in order to determine if also the training-induced increase in hepatic glycogen concentration is associated with changes in total activities of these enzymes, which are in ol ed in the control of glycogen metabolism (Hers 1976).

METHODS AND MATERIALS

Male Wistar rats weighing 90–110 g were randomly divided into 3 groups. One group was subjected to previously described (Galbo et al. 1977) 12-wk physical training program consisting of swimming 5 days wk at 33–34°C. The duration of wimming was gradually increased until after 8 wk the rats were continuously 6 h day. Sedentary control rats were either freely eating or weight matched with trained rats by restricted access to food up to 50 h before tissue sampling

All rats were anesthetized with pentothal sodium intraperitoneally in the morning after 4 h without access to food. Trained rats were used 67 h after the last wim. Liver samples were, within 1 s, removed and frozen in isopentane precooled in liquid nitrogen. The samples were stored at −80°C and analyzed within 1 wk. Homogenates ere prepared by homogenizing in Potter Elvehjem type homogenizer approximately 10 mg of frozen tissue (weighed at −20°C) suspended in 500 μl of ice cold medium. The medium contained 50 mM Tris-HCl (pH 7 4 at 23°C), 0.15% (g (100 ml)$^{-1}$) human serum albumin (KABI) and 0 6 mM dithiothreitol. Total activities (a b forms) of glycogen ynthetase and of glycogen phosphorylase in the crude homogenate were determined, the latter after 40 min incubation with 2.5 mM ATP 10 mM $MgCl_2$ and 1 U ml^{-1} purified rabbit muscle phosphorylase kinase (Sigma). Phosphorylase was assayed in the direction of glycogen (Wang & Esmann 1972) using final concentration of 66 mM uniformly labeled (^{14}C)-glucose 1-phosphate (3.8 10^6 cpm μmol^{-1}) in 100 mM PIPES (pH 6.4 at 23°C) containing in addition 7.5

Table 1 *The effect of physical training on hepatic glycogen regulating enzymes and in sex anthropometric measures*

Values are mean ± S E The number of determinations is given in parentheses. denotes value significantly different ($P<0.05$) from the corresponding value in trained rats. denotes that every single value was higher than the value in weight matched trained rat Apart from body weight ($P<0.001$) the variables in freely eating and weight matched controls rats did not differ significantly ($P>0.05$)

	Synthetase (units g)	Phosphorylase (units g)	Body weight (BW) (g)	Heart weight (% of BW)	Liver weight (% of BW)
Trained rats	3 368±0 181 (11)	10.151±0 772 (10)	364±9 (11)	0.37±0.01 (11)	3 81±0 08 (13)
Weight matched controls	[illegible]101±0 25 (4)	10.[illegible]72±0.[illegible]31 (3)	360±10 (4)	0.[illegible]9±0.01 (4)	3.[illegible] (7)
Freely eating controls	2 349±0 285 (6)	12.711±0.763 (5)	468±11 (6)	0.31±0.01 (6)	3 15±0 15 (10)

mg ml rabbit liver glycogen 100 mM NaF and 1.5 mM AMP Synthetase was assayed using 4 mM UDP-(14C)-glucose (7.5 10⁴ cpm μmol) in 50 mM Tris-HCl (pH 7 4 at 23°C) containing in addition 10 mM glucose 6-phosphate and 7 mg ml glycogen (Thomas et al 1968) The assays were performed at 30°C for 10 min One unit of activity is the amount of enzyme that incorporates 1 μmol of substrate into glycogen per min under the conditions of the assay The glycogen content was determined after hydrolysis by a hexokinase method (Karlson Diamant & Saltin 1971) Hepatic concentrations are expressed per kg wet weight (Hepatic protein concentrations were identical in the different groups of rats)

For glucose tolerance test (cf Table 3) a polyethylene catheter was inserted into a carotid artery 30 min before blood sampling Blood glucose concentrations were determined by a hexokinase method (Schmidt 1961) Data were analyzed by *t* test and the test for paired comparisons was used when applicable

RESULTS

Physically trained rats increased their body weight less than sedentary rats but developed higher relative heart weights (Table 1) The total hepatic phosphorylase activity was low r and the total synthetase activity as well as the relative liver weight were higher in trained rats than in both free ly eating and weight matched control (Table 1) A higher hepatic synthetase activity wa accompanied by a higher glycogen concentration (Table) Dur ing glucose tolerance test blood glu ose concentrations were lower in trained rats than in the other groups of rats (Table 3)

DISCUSSION

The present study has shown that physically trained rats have a higher relative liver weight and total hepatic glycogen synthetase activity and a lower phosphorylase activity than sedentary rats. In sedentary rats the measured enzyme activities agreed with previously reported values (Newman & Armstrong 1978) At the end of the experiment the training rats ate 42% more than the freely eating controls but the mentioned differences can hardly be ascribed to differences in food intake Thus since the measured hepatic parameters did not differ significantly in freely eating, sedentary rats

Table 2 *Correlation bet een hepatic glycogen and synthetase concentrations*

Two physically trained and two freely eating, sedentary control rats were randomly selected for determination of both hepatic glycogen (expressed as mmol of glucose × kg wet weight) and synthetase concentrations. Trained rats were used 67 h after they had been trained for the last time Two sedentary rat had been matched with the trained rats by restricted access to food up to 48 h before sampling. Liver samples were removed in the morning after 4 h without access to food Body weight is shown in parenthesis. Correlation coefficient =0 90[illegible] ($P<0.05$)

	Synthetase (unit g)	Glycogen (mmol kg)
Physically trained r t (363 g)	3 626	533
Physically trained rat (378 g)	3 [illegible]56	4[illegible]9
Weight matched control (368 g)	590	410
Freely eating control (51 g)	390	398
Freely eating control (490 g)	293	364
Weight matched control (371 g)	[illegible]16[illegible]	54

ble 3. *The effect of physical training on the intravascular glucose tolerance test*

m glucose concentrations ± S.E. (mmol 1 blood⁻) are shown. 125 mg glucose per 100 g body weight was injected as ... through cannulated carotid artery immediately after the 0-min blood sample had been drawn from the artery ... denotes value significantly different from the ... value significantly different from 0-min value ($P<0.05$). ... denotes value significantly different from the corresponding value in trained rats ($P<0.05$). Values in freely eating controls did not differ significantly from values in weight matched controls ($P>0.05$)

	10 min	0 min	5 min	10 min	30 min	60 min
...ned rats (n=6)	7.56±0.35	7.64±0.31	27.71±0.37*	21.41±0.45*	7.41±0.38	9.54±0.45*
...ight matched ...ntrols (n=5)	7.42±0.77	7.77±0.32	30.85±1.08*-	4.53±1.20*	15.89±1.76*-	14.56±0.10*-
...ely eating ...ntrols (n=6)	8.38±0.38	8.45±0.40	30.81±1.09*-	25.21±0.92*-	13.89±1.74*-	15.20±1.91

...mpared to sedentary rats with restricted access to ...od, although the former group ate 89% more than ...e latter. Neither can the differences in hepatic ...ta be ascribed to differences in body weight since ...ey were present in comparisons between trained ...ts and weight matched controls, and since differ...nces in weight between the two sedentary groups ...ere not accompanied by significant differences in ...epatic data.

The observed training-induced hepatic changes ...ay have several consequences. The enzyme ...hanges, the correlation between hepatic synthe...tase and glycogen concentrations and the higher ...elative liver weight in trained rats indicate an en...hanced hepatic capacity for glycogen synthesis and ...torage. Probably these changes contributed to the ...radually improved glucose tolerance which was ...ound in the trained rats and which may protect ...against the development of diabetes mellitus (Björn...torp & Sjöström 1978). However changes in ...muscular capacity to metabolize glucose may also ...improve glucose assimilation after training (Björn...torp et al. 1972). The elevation of the liver glycogen ...concentration in trained animals has the beneficial ...effect of protecting the animals against liver ...glycogen depletion and development of ...hypoglycemia, for instance during prolonged exer...cise (Baldwin et al. 1975, Galbo et al. 1977). Into the ...bargain, during submaximal exercise a higher com...bustion of fat in trained than in untrained individu...als allows a slower depletion of glycogen stores in ...the first mentioned than in the last mentioned (Baldwin et al. 1975, Holloszy & Booth 1976). The ...lower hepatic glycogen phosphorylase and the ...higher synthetase activity found in trained rats may ...together with the previously observed diminished ...hormonal response to exercise after training (Galbo et al. 1977) account for the slower depletion of liver glycogen in trained than in untrained exercising animals. The present finding of hepatic adaptive changes should encourage to further studies of metabolic adaptations to training in extramuscular tissues.

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Tachycardia during egg hypothermia in incubating ptarmigan (Lagopus lagopus)

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GABRIELSEN G & STEEN J B Tachycardia during egg-hypothermia in incubating ptarmigan (*Lagopus lagopus*). Acta Physiol Scand 1979 107 273–277 Received 13 March 1979 ISSN 0001-6772. Department of Physiology Institute of Medical Biology University of Tromsø Norway

Incubating birds regulate the egg temperature by varying their posture and the distance between eggs and brood patch In the present study we show that this homeostatic process is further assisted by varying the brood patch blood flow according to the temperature of the eggs. When female ptarmigan resume incubation of cooled eggs (e.g. after period of foraging) they immediately develop pronounced tachycardia (4 times normal in wild, 2 3 times in captive birds). Tachycardia is maintained, although at decreasing intensity until the eggs hav obtained normal temperature The eggs are heated 30 to 50% slower in females where tachycardia is inhibited by β-receptor blocking agent.

Key ords Tachycardia, incubation, ptarmigan, brood patch

Several studies ha e revealed behavioural mechanisms by which incubating birds attempt to maintain their eggs at a constant temperature during varying ambient conditions (Frith 196., Nelson 96, Drent 1970 White 1974) During a recent telemetric study on the heart rate of incubating willow ptarmigan (Gabrielsen et al 1977) data were obtained which indicated a physiological mechanism whereby the bird can ary the amount of heat transferred to the eggs It was observed that hen ptarmigan returned to incubate after foraging, their heart rates increased 4–5 fold above the resting level The data further indicated that the length of the tachycardia period increased with increasing periods aw y from the nest and lower ambient temperatures

In the present investigation w ha tested whether this tachycardia influences the rate of egg warming Our study involved observations of both wild and captive incubating ptarmigan be in an attempt to determine the causal relationsh p between egg temperature and heart rate

MATERIAL AND METHODS

The field investigation as conducted during the summers of 1976–77 and 78 on Kalbøy Troms County Norway (69°N).

A total of 8 wild willow ptarmigan hens were caught on the nest using dip nets. They were then equipped with telemetry transmitter consisting of an ECG preamplifier modulator and crystal controlled transmitter of 102.36 and 96 26 MHz With batteries for 400 h operation and transmission range of p to 200 m, the total weight of the apparatus was 23 g. The transmitter was taped to rucksack type harness One gold-tipped wire from the ECG amplifier was positioned close to the heart by means of stiff plastic tubing which had been introduced through small incision behind the sternum. The other electrode was placed under skin overlying the abdomen. Attaching the entire apparatus required about 15 min. Hens usually returned to the nest to incubate within 20 min after release, but sometimes stayed away considerably longer

Observations were made through telescope from an observation hut situated at least 40 m from the nest. The signals from the transmitter were received through direc tional Yagi antenna and commercial FM-radio and recorded on cassette tape for later analysis. Direct recording of ECG signals were made and heart rates were later derived by means of an on-line micro-computer

Experiments on captive hens were carried out at the Wildlife Research Station at the University of Tromsø Two pair of ptarmigan ere exposed to long day/short night light regime on January 1 1978 Five eeks later the birds had started to moult into breeding plumage Each pair as placed in 4 4 m indoor room arranged in natural manner ith birch branches. Food and water were

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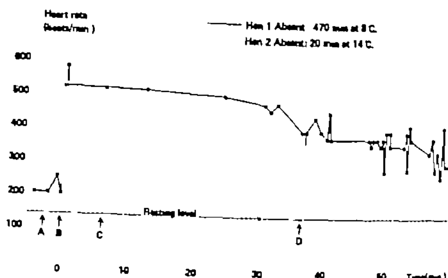


Fig 1 Heart rate of two wild incubating willow ptarmigan hens upon returning to their clutches after different periods of absence (A) Just prior to sitting on the clutch. (B) Immediately after start of incubation (C) Turning the eggs. (D) Disturbance.

given ad lib Both pairs laid a normal clutch of eggs After a week of incubation the hens were equipped with the same telemetry equipment as was used in the field The birds were placed under TV surveillance and could be observed directly as well.

For each hen an artificial clutch was assembled This consisted of 9 unfertilized ptarmigan eggs plus a temperature-measuring eggs. The latter was a blown-out ptarmigan egg shell filled with paraffin wax and fastened in a fixed position in the middle of the nest Two thermocouples were fixed inside this egg, one touching the inner surface of the shell on the uppermost part of the egg (surface egg temperature) and the other in the center of the egg (inner egg temperature)

Two series of experiments were conducted The first series consisted of exchanging the hen s own clutch with the artificial clutch which had previously been heated or cooled to a predetermined temperature When the hen returned to incubate the artificial clutch simultaneous measurements were made of the hen s heartbeat and change in egg temperature The ambient temperature was always 20– 1°C

In the second series of experiments each hen was given 0.25–0.50 mg Inderal propranolol a β-receptor blocking agent which effectively inhibits acceleration of the heart before presenting her with clutches of different temperature 4 pairs of expts. were conducted on different hens

Both hens eventually hatched their clutche

RESULTS

The field experiments revealed the following pattern of heart activity: As each hen carefully approached the nest after a period of absence the heart rates varied between 200–220 b/min. Within 10 s after settling on the eggs the heart rates rose to between 500 and 600 b/min. The duration of th tachycardia seemed to be associated with the length of time spent away from the nest and with the ambient temperatures. This is illustrated by the fol lowing examples Hen 1 (Fig. 1) stayed away for 4

Table 1 *Duration of heart rate above 500 beats min among eight wild hens returning to eggs after varying times away from the nest and varying ambient temperature*

Hen no	Ambient temp (°C)	Absence from eggs (min)	Duration of tachycardia above 500 beats/min (min)
8	17	5	1
8	15	5	1
6	15	15	1
6	15	20	1
6	16	25	3
1	14	20	3
8	15	15	3
7	9	10	3–4
1	10	15	4–5
1	10	20	4–5
4	10	20	4–5
1	10	20	5–6
1	9	20	5–6
[illegible]	8	20	5–6
5	6	20	5–6
1	7	20	5–6
1	6	20	5–6
3	4	20	6–7
1	7	145	10–11
6	8	470	20–25

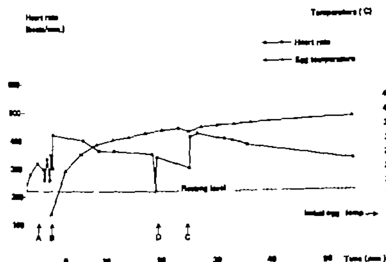


Fig. 2. Heart rate of captive hen returning to clutch of eggs cooled to 10°C and the egg temperature following incubation. A, B, C and D as in Fig. 1

and 50 min at an ambient temperature of 8°C and upon returning maintained a heart rate above 500 for 25 min. Seven hours passed before the heart rate returned to normal. Hen 2 stayed away for 70 min at an ambient temperature of 14°C. Upon returning, the heart rate increased to 575 but dropped below 300 within 5 min. After 30 min she had resumed normal heart rate (Fig. 1). During this decline period a temporary increase in heart rate occurred each time the hen had turned her eggs and a decrease occurred when she was disturbed. This latter response was described by Gabrielsen et al. (1977). Eighteen similar observations support the conclusion that this is a normal pattern of cardiac response among wild ptarmigan hens returning to their eggs (Table 1).

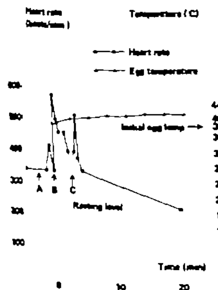


Fig. 3. Heart rate of captive hen returning to clutch of eggs with temperature of 39°C, and the egg temperature following incubation. A, B and C, as in Fig. 1

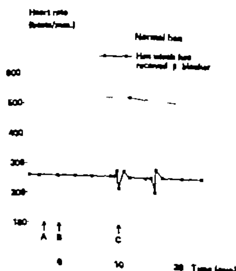


Fig. 4. Cardiac response of an inderal treated and an untreated control wild hen both incubating eggs with initial temperature of 8°C. A, B and C as in Fig. 1

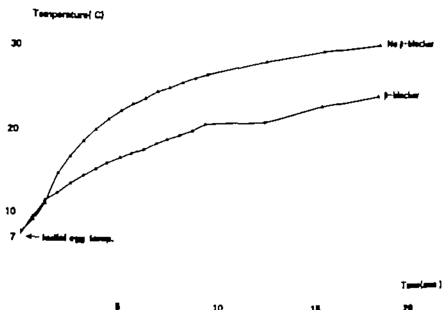


Fig 5 Sub surface egg temperature after onset of incubation by a captive hen before and after injection of β-blocker Initial egg temperature in both cases 7–8°C

Captive hens have a higher resting heart rate (?00–260 b/min) and a lower maximum heart rate (420–580 b/min) than their wild relatives (120–140 and 550–600 b/min respectively) probably due to lack of physical training.

Fig. 2 shows an example of the cardiac response of a captive hen returning to incubate a clutch which is previously cooled to 10°C The same figure also gives the surface egg temperature during the subsequent period of incubation The heart rate increased immediately and stayed high until the inner egg temperature returned to normal

Fig 3 illustrates the response of the same hen returning to eggs with a temperature of 39°C There was an initial period lasting about 5 min when the heart rate was increased but very irregular After this period it returned to normal

Fig. 4 shows that a hen which has previously received β-blocker does not display tachycardial cardiac response upon returning to 8°C eggs Her heart rate is remarkably stable at a rate ?0–30 ? above the resting level found in captive hens The same pattern was found in all the other experiments.

Fig. 5 shows that a hen which cannot accelerate her heart rate due to β-blocker will heat her eggs slower than a hen with a normal heart function Both hens began to incubate eggs cooled to 7–8°C After 5 min the normal hen had heated her eggs to 22.5°C while the other hen had heated hers to 16.5°C The results of the other experiments a compiled in Table ?

DISCUSSION

Most bird species form a brood patch, a portion of the skin covering the pectoral muscles which b comes free from feathers and richly vasculariz during incubation (Lange 1928 Allen, Stokkan & Stalsberg 1978) The incubating bird can regula the egg temperature by varying its posture and

Table 2 *Comparison of temperature increase in eggs incubated by 2 ptarmigan hens before and after they have received an intramuscular injection of the β-receptor blocking agent (propranolol)*

Hen no	Propranolol	Egg temperature (°C) Initially	After 5 min incubation	Increase
1		7 5	22.5	15 0
1		7 0	16.5	9 5
1		14 0	30.5	16 5
1	+	14 0	?6 0	1 0
		10 0	?6 0	16 0
		10 5	21.5	11 0
		?0.5	37 0	16 5
2	+	?0.0	3 0	1 0

the distance to the eggs (White & Kinney 1974) In the present study we found evidence which indicates that the heat transfer to the eggs is also regulated by variations in brood-patch blood flow Thus we showed that ptarmigan hens increase their heart-rate 2–4 fold when they return to cold eggs. This tachycardia is maintained until the eggs have attained normal brooding temperature.

Since the temperature of the eggs increased faster in hens with tachycardia, than in those where heart acceleration was inhibited by a β-blocking agent, we conclude that the blood flow to the brood patch is increased substantially during tachycardia. It is unlikely that propranolol should cause local vasodilation since arteriolar relaxation if existent, is mediated through β-receptors (Nickerson 1967) Whether the heat production of the bird is also elevated under these conditions is presently under study

The observation that heart rate increases after contact between the brood patch and eggs at a subnormal incubation temperature strongly supports the suggestion by White & Kinney (1974) that the bird senses egg temperature in the skin of the brood patch. This is further attested to by the cardiac response following the hen's turning of eggs. This behaviour inevitably exposes a cold egg surface to the brood patch and it always elicited acceleration of the heart. Signals from the receptors of the brood patch presumably activate the brood patch-thermostat in the hypothalamus which maintains tachycardia and increases egg heating as long as the eggs are below the desired brooding temperature

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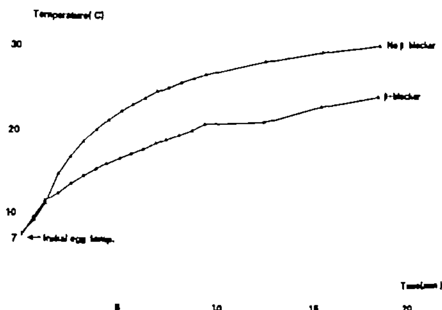


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1		7 0	16 5	9 5
1		14 0	30 5	16 5
1		14 0	6 0	1 0
		10 0	26 0	16 0
		10 5	1 5	11 0
	–	20.5	37 0	16 5
	+	20 0	3 0	1 0

Acta Physiol Scand 1979 107 279–281

Peptides in the cat carotid body (glomus caroticum) VIP enkephalin and substance P like immunoreactivity

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of Agricultural Sciences, and Department of Pharmacology Uppsala University Sweden

Recently an enkephalin-like peptide has been observed in the gland cells of the adrenal medulla, in SIF cells and in some sympathetic noradrenergic neurons (Schultzberg et al. 1978, 1979) In the present paper we report preliminary results on the occurrence of an enkephalin-like peptide as well as some other peptides in the carotid body (glomus caroticum), an organ closely related to other chromaffin systems and belonging to the so called APUD system (see Pearse 1969)

Four male or female cats (b wt. 2–4 kg) were anesthetized with Nembutal (30 mg/kg, i p) and perfused in the ascending aorta with ice cold 10% formalin (Pease 196.) for 20 min. The carotid bifurcation was rapidly dissected out and immersed in the same fixative for 90 min. Serial cryostat sections were made and processed for immunohistochemistry according to the indirect technique of Coons and collaborators (see Coons 1958) as described previously (Hökfelt et al 1973) The sections were incubated at 4°C over night with antisera to met or leu-enkephalin (dilution 1 160) VIP (1 160) or substance P (1 80) rinsed, incubated with FITC conjugated sheep anti-rabbit antiserum (SBL, Stockholm, Sweden), mounted and examined in Zeiss fluorescence microscope The antisera pretreated with an excess of the respective peptide served as control sera. For further details on preparation and characteristics of the antisera, see Schultzberg et al (1978) (enkephalin antisera) Fahrenkrug et al (1977 1978) (VIP antiserum) and Ljungdahl et al (1978) (substance P antiserum)

After incubation with both types of enkephalin antisera, strong immunofluorescence was observed in the majority of the glomus cells (Type I cells) (Fig. 1A). Often strongly fluorescent processes could be seen from the enkephalin immunoreactive glomus cells. No immunoreactive cells but many VIP immunoreactive strongly fluorescent varicose nerve fibres with a patchy distribution pattern were seen (Fig. 1B). They were found with the highest density around blood vessels, but some thin single fibres seemingly penetrated between the glomus cells. After incubation with substance P antiserum, some thin weakly fluorescent fibres were seen. None of the fluorescent structures described above were seen after incubation with control sera.

The present findings indicate that peptides are present in different structures of the carotid body Whereas an enkephalin-like peptide is present in the type I glomus cells and their processes, the VIP- and substance P-like peptides are found in nerves. These results are in agreement with the previous demonstration of an enkephalin-like peptide in other chromaffin cells (DiGiulio et al. 1978, Schultzberg et al 1978 1979). The present study does not allow an exact identification of the peptides visualized, but it is interesting that both leu- and met enkephalin as well as a larger (precursor?) molecule have been identified in sympathetic tissues (Hughes et al. 1977 DiGiulio et al. 1978).

The origin of the two types of peptide nerves in carotid body is uncertain. Since numerous substance P immunoreactive cells have been seen in the petrosal ganglion (Lundberg et al. 1979a), this peptide may be confined to peripheral sensory branches. VIP immunoreactive cell bodies have been observed in the superior cervical ganglion and may give rise to the blood vessel innervation observed (Lundberg et al 1979b).

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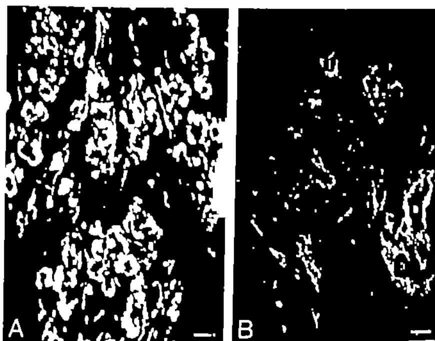


Fig 1 Immunofluorescence micrographs of the carotid body after incubation with antiserum to met-enkephalin (A) and VIP (B) (A) Numerous enkephalin immunoreactive glomus cells of type I and short processes (arrows) are seen (B) The VIP-like immunoreactivity is present in nerve fibres especially around blood vessels (b) Some fibres (arrows) seem however to run without any obvious relation to vessels. Bars indicate 50 μm

The functional significance of peptides in the carotid body remains to be elucidated The glomus cells have been ascribed several functions (see McDonald & Mitchell 1975) Our findings agree with Pearse s (1969) APUD-concept that the glomus cells are endocrine cells secreting polypeptides According to Biscoe et al (1970) the glomus cells release catecholamines to regulate the sensitivity of adjacent chemoreceptive nerve endings Perhaps the enkephalin-like peptide is involved in similar regulatory mechanism Furthermore the relation of the presence of an opioid peptide in the glomus cells to the well-known respiratory depressant action of morphine remains to be elucidated

With regard to VIP immunoreactive fibres it has been shown that intracarotid injection of VIP stimulates chemoreceptor afferents (Said & Mutt 1970) either by a direct action or possibly due to local circulatory effects The latter view is supported by the present demonstration of VIP immunoreactive nerves around blood vessels On the other hand only a very weak effect on respiration was observed after peripheral administration of substance P by the same route (McQueen 1978) It is however interesting to note that intraventricular or intracisternal administration of SP caused hyper ventilation (von Euler & Pernow 1956) and that medullary respiratory centres contain a dense net work of SP nerve terminals (Ljungdahl et al 1978)

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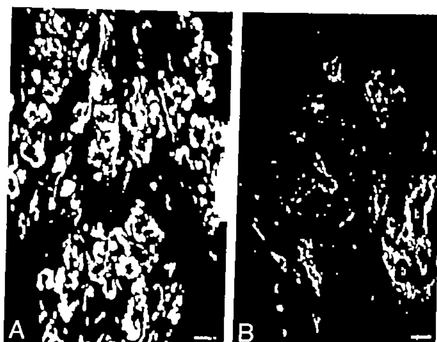


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The effect of indomethacin on the blood flow–metabolism couple in the brain under normal, hypercapnic and hypoxic conditions

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Although previous interest in the regulation of cerebral blood flow (CBF), and in the coupling of flow and cerebral metabolic rate for oxygen (CMR_{O_2}), has mainly focused on such coupling factors as extracellular H^+ and K^+, recent results have established the influence of neurotransmitters and neuromodulators. For example, it has been demonstrated that the catecholamines noradrenaline, dopamine, and adrenaline, when gaining access to intracerebral receptors, induce increases in CMR_{O_2} and CBF (MacKenzie et al. 1976*a* and *b*, McCulloch & Harper 1977, Berntman et al. 1978), and that serotonin has the opposite effects (Harper & MacKenzie 1977). It now seems that also prostaglandins exert a significant influence on CBF. Thus Pickard & MacKenzie (1973), working on phencyclidine anesthetized baboons, found that indomethacin (10 mg kg⁻¹) reduced normal CBF by about 40% and considerably curtailed the circulatory response to induced hypercapnia.

In the present study we repeated the experiments of Pickard & MacKenzie (1973) in another species under different anesthetic circumstances, and extended them to include arterial hypoxia as well. Since the results give novel information on the blood flow–metabolism couple in the brain they are reported in preliminary form.

The experiments were performed on fed male Wistar rats, weighing 320–415 g. Anesthesia was induced with 3% halothane and 70% N_2O, and continued during operative preparations on 1% halothane and 70% N_2O. The animals were tracheostomized, immobilized with tubocurarine chloride (1.5 mg kg⁻¹), and ventilated artificially by a Starling type respirator. Both femoral arteries were cannulated to allow continuous blood pressure recording and sampling of arterial blood. One femoral vein was cannulated for administration of drugs. The caudal portion of the superior sagittal sinus was exposed by means of a small burr hole to obtain cerebral venous blood. In one group of animals (see below) the adrenal glands were removed.

After the completion of operative procedures, halothane supply was discontinued and the animals were heparinized (30 I.U. kg⁻¹) and maintained on 30% O_2 and 70% N_2O (or N_2, see below) for a 30 min steady state period, rectal temperature being maintained close to 37 °C.

Control animals maintained on 70% N_2O were compared to 3 series of animals: normocapnic-normoxic, hypercapnic and hypoxic. In the first series, one group of N_2O-anesthetized animals was maintained normocapnic (Pa_{CO_2} 35–40 mmHg) and normoxic (Pa_{O_2} > 100 mmHg) for 30 min following the injection of indomethacin in a dose of 10 mg kg⁻¹ i.v. before CBF and CMR_{O_2} were measured. Since indomethacin markedly reduced CBF an interaction with N_2O had to be excluded. Therefore, indomethacin was also given to animals in which the nitrous oxide supply was discontinued for 15 min. In this group, the adrenal glands were removed and, in order to minimize pain and discomfort, the animals were given local anesthesia and protected from external stimuli. In the hypercapnic series, indomethacin (10 mg kg⁻¹ i.v.) was given 15 min before the induction of hypercapnia (Pa_{CO_2} ≈ 80 mmHg) and CBF and CMR_{O_2} were measured after 30 min of hypercapnia. Separate hypercapnic animals were studied without indomethacin. In the hypoxic series, animals were treated similarly except for the fact that hypoxia (Pa_{O_2} ≈ 25 mmHg) was induced instead of hypercapnia.

Methods for measuring CBF and blood oxygen content (Ca_{O_2}), as well as for calculating CMR_{O_2}, have been described previously (Berntman et al. 1979 *a* and *b*). These references should be consulted also for general physiological techniques. Statistical differences between groups were calculated using the unpaired Student's *t*-test.

In all experimental groups mean body temperature was close to 37°C. Changes in arterial blood pressure and pH during hypercapnia and hypoxia were similar to those previously observed under the same experimental conditions (Berntman et al. 1979*a* and *b*) and physiological parameters were similar in uninjected and indomethacin-treated animals. In

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Acta Physiol Scand 1979 107 285–287

Vasoactive intestinal polypeptide (VIP) inhibits oxytocin induced activity of the rabbit myometrium

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Vasoactive intestinal polypeptide (VIP) a highly basic octacosapeptide was isolated from porcine small intestine by Said and Mutt (1970). VIP was originally considered to be a gastrointestinal hormone but recent studies have demonstrated that VIP has an almost ubiquitous occurrence in the body localized in neurons and evidence is accumulating that the peptide represents a neurotransmitter (Fahrenkrug 1979). In the female genitourinary tract VIP-containing nerve fibres seem to innervate small vessels and smooth muscle cells (Larsson Fahrenkrug & Schaffalitzky de Muckadell 1977). This finding in connection with the potent vasodilatatory and smooth muscle relaxatory effects of VIP (Said 1975) suggest that the peptide plays a role in the nervous regulation of smooth muscle contractility and the blood flow in the genitourinary tract. Here we report that VIP inhibits the contractility and the myoelectrical activity of the myometrium in rabbits.

Five female rabbits of Danish Land race (age 140–170 days weight 4–3.0 kg) had myometrial autografts placed in preinserted bilateral transparent ear-chambers by the technique of Wagner (Wagner 1975 Wagner Hrynzuk & Nielsen 1977). Grafting was done 4–6 weeks after the ear-chamber insertion. The activity was recorded as electrical potentials by means of three platinum electrodes mounted on the base of the ear-chamber and contractions were simultaneously observed in a microscope (10–40 x magnification stereomicroscope Leitz). The experiments were performed not earlier than 10 days after grafting and the viability of the preparation was tested by its ability to respond to oxytocin intravenously which should provoke potentials of magnitude of 0.5–3 mV peak to peak (Fig. 1A). Before the experiments the animals were treated for 6 days with estradiol, 25 µg i.m. daily

On the day of experiment the rabbit was anesthetized with mebumal (Nembutal®), 30 mg/kg i.v. and a continuous infusion of oxytocin 0.3–1 mU min kg was given into an ear vein. When a stable myoelectrical response was obtained VIP was infused into the superior caval vein in the following doses in randomized order 6 pmol min kg 25 pmol min kg^{-1} 100 pmol min kg for 10-min periods. The VIP infusion periods were separated by 10 min intervals with control infusions of isotonic saline containing human serum albumin (Behringwerke Marburg am Lahn, GFR), the vehicle used for dissolving VIP

In order to estimate the concentration of VIP in the arterial blood supplying the myometrial graft VIP infusions using the protocol as above were performed on 5 additional female rabbits of identical weight and age. Blood samples were collected from the left carotid artery and the concentration of VIP measured radioimmunochemically (Fahrenkrug & Schaffalitzky de Muckadell 1977). The results are given in Table I

The highest dose of VIP used (100 pmol min kg) completely abolished the electrical activity in the myometral graft induced by oxytocin (Fig. 1C) while VIP had no effect on the non-oxytocin stimulated myometrium (not shown). A lag of 0.5 to 1.0 min was observed after discontinuation of the VIP infusion before the response to the continuous administration of oxytocin was regained. During infusion of VIP in a dose of 25 pmol min kg the myoelectrical response to oxytocin was modified and only a few spikes were recorded (Fig. 1D) while the oxytocin response was unaffected by the lowest VIP dose (6 pmol min kg) (Fig. 1E). During the period of oxytocin induced activity blanching of the graft (contraction) occurred simultaneously with the electrical potentials.

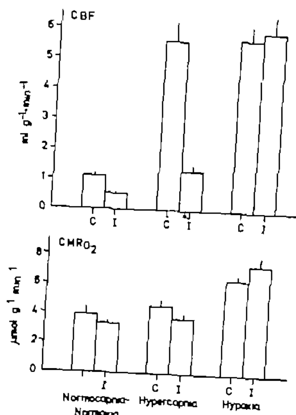


Fig 1 The influence of indomethacin on cerebral blood flow (CBF) and cerebral metabolic rate for oxygen (CMR_{O_2}) under control conditions as well as in hypercapnia and in hypoxia. Values given are mean ± S E for indomethacin injected animals (I) as compared to the corresponding control situation (C) $P<0.001$ Statistical symbols within bars indicate differences from normocapnic–normoxic controls, while those below bars indicate differences between indomethacin-injected and control groups.

all non-hypoxic groups mean Pa_{O_2} exceeded 108 mmHg and in all non hypercapnic groups mean Pa_{CO_2} varied between 35 and 39 mmHg.

Results on CBF and CMR_{O_2} are illustrated in Fig 1 In normocapnic–normoxic animals indomethacin reduced CBF to 50% of control with no influence on CMR_{O_2}. The results confirm those of Pickard & MacKenzie (1973) Similar results were obtained in animals maintained on 70% N_2 excluding an interaction between indomethacin and nitrous oxide

In uninjected hypercapnic animals CBF increased 5-fold and there was a suggested (but non significant) increase in CMR_O (cf Berntman et al 1979*a*) In indomethacin-treated animals CBF during hypercapnia was only 25% of that observed in uninjected animals, and CMR_{O_2} was close to control Also these results are in close agreement with those reported by Pickard & MacKenzie (1973)

During hypoxia in uninjected animals there was the expected marked rise in CBF and an increase in CMR_{O_2} (cf Berntman et al 1979*b*) As observed none of these changes were influenced by indomethacin In fact CMR_{O_2} in the indomethacin treated animals approached 200% of normoxic controls We are at present studying whether or not circulating catecholamines contribute to this high oxygen consumption rate (cf Berntman et al 1978

The present results allow two important conclusions First indomethacin known to inhibit prostaglandin synthesis significantly influences normal cerebrovascular tone and interferes with the coupling of metabolism and blood flow Second the drug curtails vascular dilatation during hypercapnia but not during hypoxia This suggests that the mechanisms mediating vasodilatation in hypercapnia and hypoxia are at least partly dissimilar

This study was supported by grants from the S[illegible] Medical Research Council (project No. 14X 263) and from US PHS (No 5 R01 NS07838). The authors are grateful to Gunilla Gidö for skilful technical assistance.

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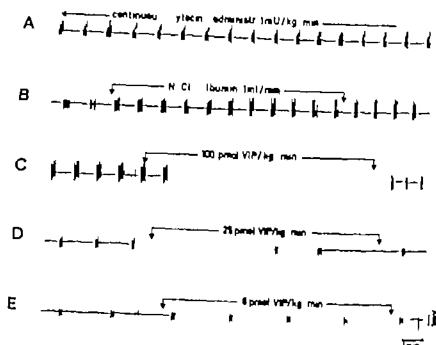


Fig 1A–E The myoelectrical activity recorded from ear-chambers in different rabbits All registrations (A–E) were preceded and followed by a 10 min long period of regular potentials similar to those illustrated at the beginning and end of each trace in the figure (A) Response to continuous oxytocin infusion (B) Control infusion of 0.5 human serum albumin (HSA) in isotonic saline (C) Response to 100 pmol VIP min kg (D) Response to 25 pmol VIP min kg (E) Response to 6 pmol VIP min kg

After initiation of the VIP infusion (100 pmol min kg) no blanching was observed and no alteration in the blood flow was visually detectable

Our results have demonstrated that VIP has a dose-dependent inhibitory action on the oxytocin activated myoelectrical and mechanical activity of the uterus The present findings and the occurrence of VIPergic nerve fibres and terminals *in the myometrium which seem to innervate the smooth* muscle cells suggest that VIP plays a physiological role as neurotransmitter in the local control of the activity of the myometrium

This work was supported by grant from the Danish Hospital Foundation for Medical Research Region of Copenhagen the Faroe Island and Greenland (J nr 87 13) and Thomas Bartholin s Fund Denmark The skilled technical assistance of Birdie Knudsen Lene Poulsen and Anita Hansen is gratefully acknowledged

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Table 1 *Plasma concentration of VIP (pmol/l) in the arterial blood supplying the myometrial graft in 5 female rabbits during infusion of VIP in graded doses*

Infusion dose of VIP pmol min kg	0	6	25	100
Concentration of VIP				
Median	18 0	59 8	45	665
Interquartile range	8 0–18 5	47 0–8_ 5	175–255	550–825

Acta Physiol Scand 1979. 107: 289–296

Quantitative changes in regional cerebral blood flow of rats induced by alpha- and beta-adrenergic stimulants

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EDVINSSON, L., LACOMBE, P., OWMAN, CH., REYNIER-REBUFFEL, A. M. & SEYLAZ, J. Quantitative changes in regional cerebral blood flow of rats induced by alpha- and beta-adrenergic stimulants. Acta Physiol Scand 1979. 107: 289–296. Received 10 April 1978. ISSN 0001-6772. Department of Histology, University of Lund, Sweden.

Cerebral blood flow was measured with the ^{14}C-ethanol technique in 8 regions (frontal, parieto-temporal and occipital cortex, caudate nucleus, thalamus, cerebellum, mesencephalon, and pons) of rats. The highest flow values (83–89.5 ml/100 g/min) were found in cortical areas, whereas pons had the lowest flow (48 ml/100 g/min). Intravenous infusion of noradrenaline or adrenaline markedly reduced rCBF (by 2...–48% of control levels) in all regions except thalamus, mesencephalon, and pons. The noradrenaline-induced reduction was blocked, and the effect of adrenaline reversed, after pretreatment with the alpha-receptor antagonist, phentolamine. Isoprenaline infusion markedly augmented rCBF in thalamus, mesencephalon, pons, and also in the caudate nucleus. The response was reduced by the beta-receptor antagonist, propranolol. The experiments show the presence and heterogeneous distribution in the cerebrovascular bed of alpha- and beta-adrenoceptors that can be activated by sympathomimetics given systemically. If noradrenaline was allowed to pass the blood-brain barrier after osmotic opening with urea, an increased regional flow was obtained, probably due to a mechanism where the vasodilator effect secondary to activation of cerebral metabolism predominated over the direct vasoconstrictor effect of the amine.

Increased emphasis has been laid on a neural contribution to the regulation of cerebral blood flow (CBF) (Edvinsson & MacKenzie 1976, Owman & Edvinsson 1977) since it could be established by modern histochemical and electron microscopic studies that the brain vascular bed receives a well-developed sympathetic innervation (see Owman, Edvinsson & Nielsen 1974). The direct net effect of the noradrenaline transmitter on the cerebrovascular smooth muscle is a constriction, as has been demonstrated in vitro (Edvinsson & Owman 1974) and by microapplication in vivo (Wahl et al. 1972).

Several studies have been undertaken to illustrate the influence of various sympathomimetic drugs on the cerebral circulation. The effects obtained have been contradictory both with regard to the direction and the amplitude of the response. Thus, the injection of noradrenaline or adrenaline has by some ... been found to decrease the flow (King, Sokoloff & Wechsler 195.., Sensenbach, Madison & Ochs 1953, Krog 1964, Häggendal 1965, von Essen 1973, Aubineau et al. 1973a, Mitchell, Scriven & Rosendorff 1975), to have no effects (Gottstein 1962, Greenfield & Tindall 1968, Olesen 1972), or by others even to induce an increase in the cerebral blood flow (Meyer et al. 1964, Biennmuller & Betz 1970, Mitchell et al. 1975). However, when considering all available data, together with a critical evaluation of the techniques involved, noradrenaline administration or cervical sympathetic stimulation reduce total resting cerebral blood flow, but only by about 10 to 15% (see review by Edvinsson & MacKenzie 1976). There are several possible reasons for the weak response. It may be the resultant of a mixture of effects in regions with prominent and with little or no flow changes (Sercombe

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et al 1975) in the case of nerve stimulation probably related to the degree of sympathetic innervation (Edvinsson & Owman 1977*a*) The poor response to some drugs may also be related to the limited passage of such substances through the blood-brain barrier (Rapoport 1976 Edvinsson et al 1978) The effect of noradrenaline might be limited by neuronal and extraneuronal uptake mechanisms preventing it from reaching the vascular adrenergic receptors (cf McCalden & Eidelman 1976)

Studies involving the beta-receptor agonist isoprenaline have given more consistent results This agent is only slightly affected by neuronal and extraneuronal uptake and degradation processes (Iversen 1967) The common response to systemically administered isoprenaline is vasodilation and increase in the cerebral perfusion (Laubie & Drouillat 1867 Xanalatos & James 1977 Aubineau et al 1973*a* and *b*)

The adrenoceptors in cerebral blood vessels and their pharmacological characteristics have been extensively analyzed (Edvinsson & Owman 1977*b*) Wahl & Kuschinsky 1977) Based on the recent findings of local differences in the perivascular sympathetic innervation (Edvinsson & Owman 1977*a*) and in the reactivity to vasoactive amines and to sympathetic nerve stimulation (Sercombe et al 1975) the present series of expts were performed to analyze in detail the changes in regional cerebral blood flow of rats induced by alpha and beta adrenergic receptor agonists *alone* or in combination with specific competitive antagonists

METHODS

Animals The experiments were performed on adult male Sprague-Dawley rats (250–300 g b wt) having free access to tap water and commercial pellet food until operation Anesthesia was induced with halothan (Fluothane Hoechst –3%) delivered via a Drager vaporizer When asleep the animals were tracheotomized and paralyzed (0.5 mg/kg tubocurarine chloride) Thereafter they were ventilated with a small animal respirator (Braun) on 70% N_2O and 30% O_2 without halothane Body temperature was measured rectally and adjusted to 37°C by a heating lamp The femoral arteries and veins were cannulated bilaterally One of the arterial catheters was used for continuous recording of arterial blood pressure via pressure transducer (Elema, Sweden) and the other for anaerobic sampling of blood both for blood gas measurements (BMS Mk II Radiometer Copenhagen) and for sampling of radioactive tracer One of the femoral veins was cannulated for infusion of the radioactive tracer [14]C ethanol (specific activity 60 mCi/mmol Radiochemical Centre Amersham) 20 μCi dissolved in physiolog saline and the other femoral vein for administration the drugs to be tested and of saturated potassium chlori to stop the heart at the end of the expts.

CBF measurements The flow determinations were not performed until the p_aCO_2 had stabilized between and 4 mmHg for at least 30 min. The regional blo flow was determined according to Reivich et al. (19 as modified by Eklöf et al (1973) The [14]C-ethanol sol tion was injected into the circulatory bed via a femor vein during 30 s using a constant rate infusion pum Arterial blood samples were taken into 10 μl glass cap laries from a femoral artery every 3 s during the isoto infusion

Immediately after the last sample had been taken, 1 ml of saturated potassium chloride was rapidly injected t stop the heart. The animal was instantaneously decap tated the head frozen in toto in liquid nitrogen, and store at −25°C in a cryostat for later preparation (1–4 days). Th brain was removed and various regions dissected out in cryostat at a temperature of −15°C where also the tiss pieces were weighed on a microbalance (Kahn). The encephalic samples taken for analysis consisted mainly grey matter These pieces of brain tissue as well as th previously withdrawn blood samples were added to scin tillation vials and counted in a Nuclear Chicago liqui scintillation counter The blood samples were mixed di rectly with scintillation fluid (Instagel Radiochemica Centre Amersham) while the tissue pieces were dis solved in Soluene (Packard) before the scintillation fluid was added Quenching was corrected according to stan dard procedures (see Eklöf et al. 1973)

The partition coefficient for [14]C-ethanol has been de termined by Eklöf et al (1977) and found to be 1 14 for th brain tissue Calculations of the flow values were per formed as described by Reivich et al. (1969). The flow values were computed on a Wang desk computor accord ing to the protocol used by Eklöf et al. (1973), and the results are expressed on the basis of tissue wet weight i ml/100 g/min. The CO_2 responsiveness was tested by ad ing small quantities of CO_2 to the gas mixture delivere by the ventilator until the desired level of arterial CO_2 concentration was reached

The results are expressed as mean±SE and the differ ences between mean values were analyzed statisticall with the Student's t test using a Packard desk computer

Drugs The agonists (L-arterenol hydrochloride Sigma L-epinephrine bitartrate Sigma L isoproterenol hydro chloride Sigma) were infused i v during a 10 min period with LKB roller pump at a speed of 0.1 ml/min The inhibitors phentolamine (Regitin, Ciba) and prop ranolol (Inderal Scanmeda) were administered i just before infusion of the agonist was started All drugs were dissolved in 0 9% NaCl and prepared fresh. The catecholamine solutions contained 0 2 mg/ml ascorbic acid to minimize oxidation of the amine

RESULTS

Physiological parameters The mean values of the physiological variables during the infusion expts.

Table 1. Mean arterial blood pressure and arterial blood gases and pH in anesthetized at in kich CBF as affected by infusion of noradrenaline adrenaline or isoprenaline

Mean values ± S.E. number of animals

Experimental groups		MABP (mmHg)	p_aCO_2 (mmHg)	p_aO_2 (mmHg)	pH (mmHg)
Control animals	17	144± 4	39.3±1.6	138± 6	7.36_ _0.016
Noradrenaline	6	174± 7	37.6±0 6	151_ 7	7.394 0.008
Adrenaline	6	117±14	36.8±1.5	158± 7	7.388±0 006
Isoprenaline	6	160_ 7	42.0 1.3	135 11	7.255±0.016
Phentolamine noradrenaline	5	152± 4	42.1±1 0	148±11	7.346±0 030
Phentolamine + adrenaline	5	150± 4	39 2±0.4	156± 1	7 397_0 0_
Propranolol + isoprenaline	4	140± 5	39.8±0 8	149_ 6	7.3_5±0 014

are shown in Table 1. No major differences in pH or arterial blood gases (p_aO_2 and p_aCO_2) were found in comparison with the untreated control animals. There was a statistically insignificant reduction in MABP during the adrenaline infusion whereas noradrenaline increased the blood pressure by about 20% ($P<0.01$).

Cerebral blood flow. The mean CBF values (ml/100 g/min) varied according to the region studied: the cerebrocortical grey substance usually had higher flow (frontal cortex 84, parieto-temporal cortex 89.5, occipital cortex 83) than those preparations containing less grey matter (pons 48, cerebellar cortex 62.5). Deep grey regions, such as the caudate nucleus (75.5) and the thalamus (74) had intermediary flow levels, all in good agreement with other studies (Reivich et al. 1968, Eklof et al. 1973).

Reactivity to hypercapnia. The cerebrovascular reactivity to increased arterial CO_2 tension was examined in the cerebrocortical areas and in the caudate nucleus. A modest hypercapnia (p_aCO_2 approximately 53 mmHg) was induced by adding CO_2 gas to the inspired anesthetic gas mixture of N_2O and O_2. Hypercapnia markedly increased the blood flow (Table 2) in the pooled cortical areas ($P<0.01$) and in the caudate nucleus ($P<0.001$). The CO_2 reactivity (expressed as flow increment per unit increase in p_aCO_2 above control) varied between _6 and 5.5 ml/100 g/min for each mmHg in these expts. Whether the reactivity really is higher in the caudate nucleus than in the cortex is difficult to establish on the basis of the present figures since neither the difference between control flows nor that between the hypercapnic flow values in the two areas was statistically significant.

Infusion of noradrenaline. Continuous i.v. infusion for 10 min of noradrenaline (1 µg/kg/min) before measuring the regional CBF resulted in a variety of flow responses (Fig. 1). Thus, the amine produced no significant effect in thalamus, mesence-

Table 2. Flow in cortex (mean from frontal parieto-temporal and occipital) and caudate nucleus together with values for p_aCO_2 during hypercapnia as compared with the effect of noradrenaline infusion before and after opening of the blood-brain barrier with urea

Flow values corrected to control levels of p_aCO_2 are also given. Figures show mean values ± S.E., number of animals within parenthesis. Comparison of mean values (control group vs. experimental groups) according to Student's t-test: $P<0.05$, $^{**}0.001<P<0.01$, $^{***}P<0.001$

Treatment	Cortex blood flow (ml/100 g/min)	Caudate nucleus blood flow (ml/100 g/min)	p_aCO_2 (mmHg)
Control animals	85±6 7 (18)	75±5(6)	39.3±1.6
Hypercapnia	122±8 (15)	15_ _10*** (6)	53.3±2 5
Noradrenaline i.v. infusion	66±5 3* (18)	55±5.5 (6)	37.6±0 6
Corrected to control p_aCO_2	62±5.3	46±5 5**	
2 M urea followed by noradrenaline*	117±9 7* (23)	124±11** (8)	36.2±1.0
Corrected to control p_aCO_2	109±9 7*	107±11**	

* Data calculated from material presented by Edvinsson et al. (1978).

et al 1975) in the case of nerve stimulation probably related to the degree of *sympathetic* innervation (Edvinsson & Owman 1977*a*) The poor response to some drugs may also be related to the limited passage of such substances through the blood brain barrier (Rapoport 1976 Edvinsson et al 1978) The effect of noradrenaline might be limited by neuronal and extraneuronal uptake mechanisms preventing it from reaching the vascular adrenergic receptors (cf McCalden & Eidelman 1976)

Studies involving the beta receptor agonist isoprenaline have given more consistent results This agent is only slightly affected by neuronal and extraneuronal uptake and degradation processes (Iversen 1967) The common response to systemically administered isoprenaline is vasodilation and increase in the cerebral perfusion (Laubie & Drouillat 1867 Xanalatos & James 1972 Aubineau et al 1973*a* and *b*)

The adrenoceptors in cerebral blood vessels and their pharmacological characteristics have been extensively analyzed (Edvinsson & Owman 1977*b*) Wahl & Kuschinsky 1977) Based on the recent findings of local differences in the perivascular sympathetic innervation (Edvinsson & Owman 1977*a*) and in the reactivity to vasoactive amines and to sympathetic nerve stimulation (Sercombe et al 1975) the present series of expts were performed to analyze in detail the changes in regional cerebral blood flow of rats induced by alpha- and beta-adrenergic receptor agonists alone or in combination with specific competitive antagonists

METHODS

Animals The experiments were performed on adult male Sprague Dawley rats (250–300 g b wt) ha ing free access to tap water and ommercial pellet food until operation Anesthesia was induced with halothane (Fluothane Hoechst –3%) delivered via Drage porizer When asleep the animals were tracheotomized and paralyzed (0.5 mg/kg tubocurarine chloride i) Thereafter they were entubated with a small animal respirator (Braun) on 70% N_2O and 30% O without halothane Body temperature was measured rectally and adjusted to 37°C by heating lamp. The femoral arterie and ein were cannulated bilaterally One of the arterial catheters was used for continuous recording of arterial blood pressure a pressure transducer (Elema, Sweden) and the other for anaerobic sampling of blood both for blood ga me sure ments (BMS Mk II Radiometer Copenhagen) and for sampling of radioactive tracer One of the femoral veins was cannulated for infusion of the radioactive tracer ¹⁴C ethanol (specific activity 60 mCi/mmol Radiochemical Centre Amersham) 20 μCi dissolved in physiologic saline and the other femoral vein for administration the drugs to be tested and of saturated potassium chlori to stop the heart at the end of the expts.

CBF measurements The flow determinations we not performed until the p_aCO_2 had stabilized between and 42 mmHg for at least 30 min. The regional blo flow was determined according to Reivich et al. (196 as modified by Eklöf et al. (1973) The ¹⁴C-ethanol sol tion was injected into the circulatory bed via a femor vein during 30 s using a constant rate infusion pum Arterial blood samples were taken into 10 μl glass cap laries from a femoral artery every 3 s during the rota infusion.

Immediately after the last sample had been taken, I ml of saturated potassium chloride was rapidly injected stop the heart. The animal was instantaneously decap tated the head frozen in toto in liquid nitrogen, and stor at −45°C in a cryostat for later preparation (1–4 days) Th brain was removed and various regions dissected out in cryostat at a temperature of −15°C, where also the tiss pieces were weighed on a microbalance (Kahn). The te encephalic samples taken for analysis consisted mainly grey matter These pieces of brain tissue as well as th previously withdrawn blood samples were added to scin tillation vials and counted in a Nuclear Chicago liqu scintillation counter The blood samples were mixed di rectly with scintillation fluid (Instagel Radiochemical Centre Amersham) while the tissue pieces ere dis solved in Soluene (Packard) before the scintillation flu was added. Quenching was corrected according to stan dard procedures (see Eklöf et al 1973)

The partition coefficient for ¹⁴C-ethanol has been de termined by Eklöf et al (1972) and found to be 1 14 fo brain tissue Calculations of the flow alues were per formed as described by Reivich et al. (1969). The flow values were computed on a Wang desk computor accord ing to the protocol used by Eklöf et al. (1977), and the results are expressed on the basis of tissue wet eight a ml/100 g/min. The CO_2 responsivene s was tested by add ing small quantities of CO_2 to the gas mixture delivere by the ventilator until the desired level of arterial CO_2 concentration was reached

The esults are expressed as mean ± SE. and the differ ence between mean values were analyzed statistically with the Stude t s t test using a Packard desk computer

Drug The agonists (L arterenol hydrochloride Sigma L-epinephrine bitartrate Sigma, L isoproterenol hydro chloride Sigma) were infused i during a 10 min period with a LKB roller pump at a speed of 0.1 ml/min The inhibitors phentolamine (Regitin Ciba) and propranolol (Inderal Scanmeda) were admin stered I just before infusion of the agonists was started. All drugs ere dissol d in 0 9% NaCl and prepared fresh. The catecholamine solutions contained 0 2 mg/ml ascorbic acid to minimize oxidation of the amine.

RESULTS

Physiological parameters The mean values of the physiological variables during the infusion expts

increase in the cortical flow was found ($P<0.01$) in contrast to the reduction obtained with noradrenaline alone (Table ?). The flow increment was even more pronounced in the caudate nucleus ($P<0.01$) The increase was somewhat less prominent in both regions after correction to control values for p_aCO_2.

Infusion of adrenaline The pattern of response to adrenaline infusion (1 μg/kg/min) was similar to the changes in CBF found with noradrenaline (Fig. 1) Thus, no statistically significant effect was obtained in pons, thalamus, mesencephalon. The mean reduction in the caudate was the same as with noradrenaline, but due to a greater individual variability in the response the adrenaline effect was not statistically significant. In the other regions studied the vasoconstrictor response to this amine was more pronounced than for noradrenaline giving a 44–48% reduction of flow compared with untreated controls.

Administration of the alpha-receptor blocking agent, phentolamine (1 mg/kg i.) 30 min before the start of the adrenaline infusion totally inhibited the vasoconstrictor effect the vascular response was instead markedly reversed (Fig. 1). A highly significant increase ($P<0.001$) in flow compared to controls thus occurred in pons and thalamus (92 and 47% flow increment, respectively) as well as in the caudate nucleus and mesencephalon (45–46%).

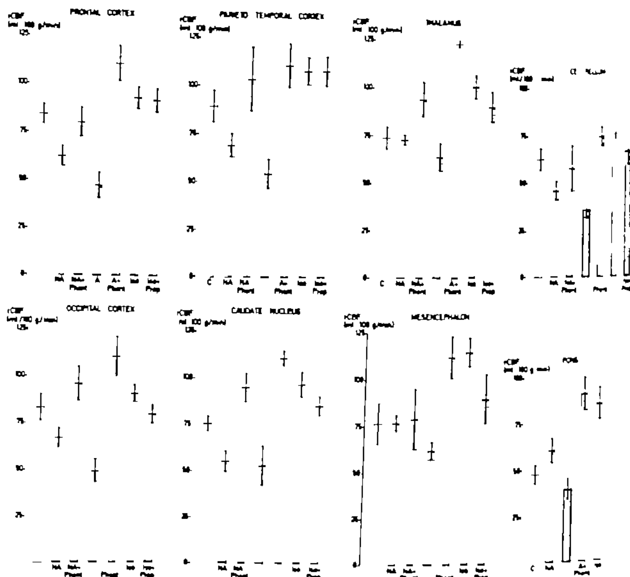
Infusion of isoprenaline In order to obtain more direct information about the vascular response to beta-receptor activation, isoprenaline (0.5 μg/kg/min) was tested during steady-state conditions (Fig. 1). Clearcut increases in regional blood flow was found in pons, mesencephalon, thalamus and caudate nucleus. The cortical regions and cerebellum only showed tendency to flow increase which was not statistically significant.

The values for p_aCO_2 in this group of animals was higher than in the controls. Correction of the p_aCO_2 levels to control values tended to reduce the figures for the flow increment. I v injection of 1 mg/kg of the beta-receptor blocking agent, propranolol, antagonized the response to isoprenaline in cases where this had produced a clearcut flow increase (Fig. 1).

DISCUSSION

Methodological onsiderations In order to obtain information on the effects of various amines on the cerebral blood flow in small animals, regional method should be used which is atraumatic with regard to the head region and, ideally it should neither require exposure of the brain tissue by means of a craniotomy nor should it disturb the local circulation of the tissue by direct injections. A technique fulfilling these criteria has been devised by Reivich et al (1969) This method is based on the mathematical calculations once derived by Kety (1951) using the diffusible tracer [14]C-antipyrine. It has later been applied to the rat and modified since it did not give adequate quantitative information in rapid flow situations (Eklöf et al. 1973). Provided the more freely diffusible tracer [14]C-ethanol is used and the infusion is carried out during 30 s, the method gives accurate values within the range of flow studied in the present experiments (Eklöf et al 1973)

As with all clearance methods the animal must be in a steady state during at least the period when the clearance is recorded. Therefore, continuous infusion of the test substance was used beginning 10 min before the start of the injection of [14]C ethanol. Although the method is regional it can be assumed that the pieces, weighing about 25 mg for practical reasons, are composed of several areas with different flow characteristics under normal and experimental conditions. It is an advantage though, that the regions measured can be exactly defined (in contrast to situations when radioactivity is measured externally) and that any extracranial contamination is entirely eliminated The control blood flow levels obtained in the present study on 250–300 g male Sprague-Dawley rats were somewhat lower than those reported for 300–400 g male Wistar rats (Eklöf et al. 1973) indicating hemodynamic variations between the two strains of highly inbred rats. The mean arterial blood pressure was affected to a varying but small extent by the different infused drugs. It has, however been shown in numerous studies (e.g. Häggendal & Johansson 1965 Harper 1966) that the autoregulation of the cerebral circulation efficiently compensates for this. Therefore, the effects noted after the amine infusions cannot be secondary to the blood pressure changes. Attempts were made to maintain blood gases (p_aCO_2 and p_aO_2) and pH within normal limits in the various experimental groups. Despite this effort, slight variations were noted in the mean p_aCO_2 (as well as p_aCO_2 and pH) values in some of the groups. For this reason, flow levels were also given corrected to control p_aCO_2. The same tendency in the flow



Regional cerebral blood flow (rCBF) in untreated control rats (C) and after infusion of catecholamines (NA noradrenaline A adrenaline ISO isoprenaline) alone or after pretreatment of the animals with competitive antagonists (Phent phentolamine Prop propranolol) Mean values ±S E For information about the number of animals and the associated physiological parameters see Table 1

phalon and pons In all the other regions the flow was reduced by approximately 10 to 27% The over all reduction in (uncorrected) cortical blood flow amounted 22% ($P<0.05$) As shown in Tables 1 and 2 there was a slight (though statistically insignificant) fall in p CO_2 in conjunction with the noradrenaline infusions Assuming a linear relationship between the flow and arterial CO pressure increments in the interval tested (39 to 53 mmHg) the CBF levels could be corrected to the same (control) p CO_2 value This slightly augmented the noradrenaline induced reduction in both cortical and caudate blood flow

The reduction in local cerebral blood flow to noradrenaline could be prevented by the injection of the alpha receptor antagonist phentolamine (1 mg/kg i v) prior to the infusion of the amine (Fig 1) There was even an increase in the flow under these conditions The reversal was statistically significant ($P<0.05$ and $P<0.001$) in all regions except the parieto-temporal cortex and the cerebellum If noradrenaline was administered after the structural blood-brain barrier had been opened through a hyperosmotic shock (see Edvinsson et al 1978) by injection of M urea (which in itself did not significantly affect CBF) into the internal carotid an

crease in the cortical flow was found ($P<0.01$) in contrast to the reduction obtained with noradrenaline alone (Table 2). The flow increment was even more pronounced in the caudate nucleus ($P<0.01$). The increase was somewhat less prominent in both regions after correction to control values for p_aCO_2.

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changes during the various treatments was found after this correction. No corresponding correction was made for variations in p O_2 values since it is known that as long as p O_2 is kept above 100 mmHg there is no significant influence on CBF (Borgström, Johansson & Siesjö 1975).

Infusion of sympathomimetic amines. In order to achieve steady state conditions continuous infusion of the agonists was utilized. Both noradrenaline and adrenaline reduced the regional flow in cerebral and cerebellar cortical structures to a varying degree and a reduction was also observed in the caudate nucleus. A similar pattern of decrease in regional CBF has been reported during cranial sympathetic nerve stimulation in rabbits (Lacombe et al. 1977).

The finding on the caudate nucleus confirms results from local measurements with the thermoclearance technique in rabbits where these amines as well as sympathetic nerve stimulation clearly reduced the caudate blood flow (Sercombe et al. 1975). During those experiments another brain region, the lateral geniculate body, showed little or no response. The difference in the response could be related to the degree of vascular innervation: the caudate has a density of sympathetic nerve supply which is twice as high as that found in the vascular bed of the lateral geniculate body (Sercombe et al. 1975). This regional heterogeneity in the extent of sympathetic nerve supply of intracerebral arterioles has been studied in more detail in baboons (Edvinsson & Owman 1977*a*). It may be that this kind of variability to some extent explains the different regional flow reactions seen also in rats (see further below).

The CBF reduction obtained after administration of the catecholamines was mediated by alpha-receptors in the sense that it could be prevented by previous injection of the competitive alpha-antagonist phentolamine. It is notable that the reduction in CBF is a net effect including a vasodilatory component as shown by the reversal, particularly of the adrenaline induced flow reduction, upon administration of phentolamine. This supports previous evidence (Edvinsson & Owman 1974, Sercombe et al. 1977) that direct cerebral vasodilatation is mediated by the $beta_1$ subtype of receptors in the vessel wall (whereas peripheral vasodilatation is known to be mediated by $beta_2$-receptors). Regions which previously had not responded significantly to noradrenaline or adrenaline (i.e. thalamus, mesencephalon and pons) now showed a marke flow increase. This shows that vasoconstrictor an dilator responses to catecholamines had outbal anced each other in the absence of phentolamine. This might reflect a regional heterogeneity in th relative proportion of constrictory and dilatory ad renoceptors, perhaps related to a local variation i the degree of sympathetic innervation as discusse above. A similar large increase in flow was ob served after administration of the beta-agonis isoprenaline. The effect was antagonized by th competitive beta-receptor blocking agent, prop ranolol.

There is reason to believe that the above men tioned effects of the sympathomimetic amines o CBF primarily represent direct vasomotor action in this vascular bed. Amines are known to penetrat the blood-brain barrier poorly (see Rapoport 1976 but can be made to reach into the brain by transie opening of the barrier with hypertonic solutions, a shown in the rat by Edvinsson et al. (1978). Unde these conditions, however, noradrenaline increase local CBF by an effect that was not inhibited b phentolamine. This effect was therefore due t activation of intracerebral neurons (MacKenzie e al. 1976*a*) mediated by beta-receptors (Edvinsso et al. 1978) causing an increase in brain metabolis and, secondarily, an enhancement of local bloo flow.

It has been claimed by Rosendorff & collab orators (1976), on the basis of intracerebral an systemic administration of amines, that the effect on intracerebral vessels are mediated throug beta-adrenergic receptors in the vascular wall, whereas the sympathomimetic effects on extra cerebral (pial) vessels are mediated by vascula alpha-receptors. In our opinion both vascular bed are supplied with alpha- as well as beta-adrenergi receptors: the effect of an exogenous amine or o noradrenaline released from perivascular sym pathetic nerves represents the net effect of simul taneous alpha-receptor mediated constriction and beta-receptor mediated dilation in the regio studied. Moreover, regional variation in the sym pathomimetic response may also be associated with the different degree of sympathetic perivascular in nervation in the brain (Edvinsson & Owman 1977*a* Sercombe et al. 1975). When noradrenaline is liber ated from the intracerebral neuron system, or when it is administered directly into the brain (com parable to situations where the blood-brain barrier

bypassed or circumvented during systemic administration), it increases blood flow through an indirect action, ie a beta-receptor mediated increase in the metabolism of those cerebral neurons that are innervated by the catecholaminergic nerve endings (MacKenzie et al. 1976*a* and *b* Edvinsson et al. 1978).

Financial support from the S edish Medical Research Council (grant No. 04X 732), the Swedish Natural Science Research Council, and from Centre National de la Recherche Scientifique.

The skilful technical assistance of Ulla-Britt Andersson and Cora Holtman is gratefully acknowledged.

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The finding on the caudate nucleus confirms results from local measurements with the thermoclearance technique in rabbits where these amines as well as sympathetic nerve stimulation clearly reduced the caudate blood flow (Sercombe et al 1975) During those experiments another brain region the lateral geniculate body showed little or no response The difference in the response could be related to the degree of vascular innervation the caudate has a density of sympathetic nerve supply which is twice as high as that found in the vascular bed of the lateral geniculate body (Sercombe et al 1975) This regional heterogeneity in the extent of sympathetic nerve supply of intracerebral arterioles has been studied in more detail in baboons (Edvinsson & Owman 1977*a*) It may be that this kind of variability to some extent explains the different regional flow reactions seen also in rats (see further below)

The CBF reduction obtained after administration of the catecholamines was mediated by alpha receptors in the sense that it could be prevented by previous injection of the competitive alpha antagonist phentolamine It is notable that the reduction in CBF is a net effect including a vasodilatory component as shown by the reversal particularly of the adrenaline induced flow reduction upon administration of phentolamine This supports previous evidence (Edvinsson & Owman 1974 Sercombe et al 1977) that direct cerebral vasodilatation is mediated by the beta subtype of receptors in the vessel wall (whereas peripheral vasodilatation is known to be mediated by $beta_2$-receptor) Regions which previously had not responded significantly to noradrenaline or adrenaline (i e thalamus mesencephalon and pons) now showed a marked flow increase This shows that vasoconstrictor and dilator responses to catecholamines had outbalanced each other in the absence of phentolamine This might reflect a regional heterogeneity in the relative proportion of constrictory and dilatory adrenoceptors perhaps related to a local variation in the degree of sympathetic innervation as discussed above A similar large increase in flow was observed after administration of the beta-agonist isoprenaline The effect was antagonized by the competitive beta-receptor blocking agent, propranolol

There is reason to believe that the above-mentioned effects of the sympathomimetic amines on CBF primarily represent direct vasomotor actions in this vascular bed Amines are known to penetrate the blood-brain barrier poorly (see Rapoport 1976), but can be made to reach into the brain by transient opening of the barrier with hypertonic solutions as shown in the rat by Edvinsson et al (1978). Under these conditions however noradrenaline increased local CBF by an effect that was not inhibited by phentolamine This effect was therefore due to activation of intracerebral neurons (Mackenzie et al 1976*a*) mediated by beta-receptors (Edvinsson et al 1978) causing an increase in brain metabolism and secondarily an enhancement of local blood flow

It has been claimed by Rosendorff & collaborators (1976) on the basis of intracerebral and systemic administration of amines that the effects on intracerebral vessels are mediated through beta adrenergic receptors in the vascular walls whereas the sympathomimetic effects on extracerebral (pial) vessels are mediated by vascular alpha receptors In our opinion both vascular beds are supplied with alpha as well as beta adrenergic receptors the effect of an exogenous amine or of noradrenaline released from perivascular sympathetic nerves represents the net effect of simultaneous alpha receptor mediated constriction and beta receptor mediated dilation in the region studied Moreover regional variation in the sympathomimetic response may also be associated with the different degree of sympathetic perivascular innervation in the brain (Edvinsson & Owman 1977*a* Sercombe et al 1975) When noradrenaline is liberated from the intracerebral neuron systems or when it is administered directly into the brain (comparable to situations where the blood-brain barrier

bypassed or circumvented during systemic administration), it increases blood flow through an direct action, via a beta-receptor mediated increase in the metabolism of those cerebral neurons that are innervated by the catecholaminergic nerve endings (MacKenzie et al. 1976a and b Edvinsson et al 1978).

Financial support from the Swedish Medical Research Council (grant No 04X 732), the Swedish Natural Science Research Council, and from Centre National de la Recherche Scientifique.

The skilful technical assistance of Ulla-Britt Andersson and Cora Bultman is gratefully acknowledged.

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Acta Physiol Scand 1979 107 297–308

Inhibitory actions from low and high threshold cutaneous afferents on groups II and III muscle afferent pathways in the spinal cat

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JENESKOG, T Inhibitory actions from low and high threshold cutaneous afferents on groups II and III muscle afferent pathw y in the spinal cat. Acta Physiol Scand 1979 107 297–308 Received 16 Oct 1978 ISSN 0001-6772. Department of Physiology Univer ity of Umeå, Sweden

The inhibitory effects caused by olley in cutaneous afferents on the transmission through some polysynaptic segmental pathways activated by high threshold muscle afferents were studied in chloralose anesthetized, spinal cats. Pathways studied were groups II and III to motoneurones as well as group II to primary afferents. The results suggested that two different mechanisms were involved. One mechanism, with a ery slow time course (duration mor than 400 ms) is suggested to be an example of presynaptic inhibition between different primary afferent systems. This mechanism required high threshold (≥1.6T) conditioning shocks, and appeared simultaneously with the component II dorsal root potential being evoked by the cutaneous afferent volley The other mechanism, with a faster time course (duration always below 300 ms), was dependent pon low threshold (≤1.5T) cutaneous conditioning volleys This inhibitory interaction could not be ascribed to the same presynaptic mechanism, but is suggested to be an example of postsynaptic inhibition at an interneuronal level. The presumed disynaptic excitatory pathway from group II muscle afferents to flexor motoneurones was not inhibited by cutaneous conditioning shocks, but could on the contrary be facilitated by activity in low threshold cutaneous afferents, probably at the only interneurone involved in this group II pathway

Key ords Cutaneous afferents, reflex path transmission, presynaptic inhibition, postsynaptic inhibition, spinal cat

Inhibition of transmission through segmental polysynaptic reflex pathway to alpha-motoneurones and to primary afferent terminals may be achieved in several ways Four main types may be considered: inhibitory interaction between segmental paths, which can be postsynaptic or presynaptic, and inhibition by descending systems, which again can be postsynaptic or presynaptic (see Eccles 1964 Lundberg 1966, 1969 1975 Schmidt 1973 for reviews) There is also the possibility of spatial facilitation between descending and segmental systems for postsynaptic interaction (Lundberg 1975) as well as for presynaptic interaction (e g Carpenter et al 1963*b* Hongo et al. 1972)

One particular descending path, the dorsal reticulospinal system which gives longlasting postsynaptic inhibition to first order interneurones in a number of polysynaptic pathways (to alpha-motoneurones to primary afferent terminals and to certain ascending systems) has been particularly well analyzed (Engberg et al. 1968*a* *b*). This descending system was recently identified as the rubro-bulbospinal path (RBSP) by Jeneskog & Johansson (1977) These authors found further more that low intensity stimulation of distal cutaneous nerves also inhibited the segmental reflex transmission in a very similar way and it was even suggested that the RBSP and the distal, low threshold cutaneous afferents may act through a common group of interneurones which postsynaptically inhibit interneurones in the segmental polysynaptic reflex pathways

The inhibition from cutaneous afferents of polysynaptic reflex pathways has now been further

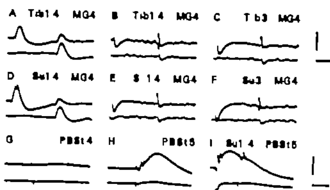


Fig 1A–F Low and high threshold cutaneous inhibition of Group II EPSP in a PBSt motoneurone Each pair of traces (4 averaged) show test EPSP alone (lower) and conditioned EPSP (upper) (A) Low threshold conditioning stimulation of Tib nerve 50 ms interval (B) Same as A but 260 ms interval (C) High threshold conditioning stimulation of Tib nerve ?60 ms interval (D–F) Same as A–C but conditioning stimulations to Su nerve. (G–I) Low threshold cutaneous inhibition of Group II DRP evoked by stimulation of PBSt nerve. Each pair of traces (8 averaged) show DRP in an S1 filament (upper) and incoming volley in L7 (lower) (G–H) Stimulation of PBSt nerve at 4T and 5T respectively (I) Suppression of Group II DRP by preceding stimulation of low threshold afferents in Su nerve 30 ms interval Calibrations at C horisontal bar 20 ms to A and D 100 ms to B–C and E–F vertical bar (positivity upwards) 4 mV to A–F Calibrations at I horisontal bar 20 ms to G–I vertical bar (negativity upwards only for upper traces) 0 4 mV

investigated The results suggest that a pathway from low threshold cutaneous afferents may inhibit polysynaptic segmental reflex pathways from muscle afferents (groups II and III) to alpha-motoneurones and to primary afferent terminals It is suggested that this inhibition is exerted at an interneuronal level but there are also results suggesting inhibition by a presynaptic mechanism in agreement with earlier studies (Eccles et al 1962*b*) This mechanism works however only when also high threshold cutaneous afferents are activated by the conditioning volley

METHODS

The results to be described were collected from 10 cat weighing ..–4 0 kg. The initial procedures and the care of the animals during the experiments were similar to those described previously (Jeneskog & Johan son 1977) During recording the animals were paralyzed with Flaxedil (Pharma Rhodia) and artificially ventilated.

Operation Two laminectomies were performed one exposing the lower thoracic cord (Th1 Th13) where the dura was opened and the spinal cord transected, the other exposing the lumbar enlargement. In some experiment thin dorsal rootlets from caudal L6 and/or rostral S1 were dissected for about 15 mm and cut distally for later recording of dorsal root potentials.

Some or all of the following nerves in the left hind limb were dissected and cut peripherally (abbreviations in parentheses): anterior biceps and semimembranosus (ABSm) posterior biceps and semitendinosus (PBSt), gastrocnemius and soleus (GS) nerve to pretibial flexors (DP) sural (Su) cutaneous division of superficial peroneal (SP) and tibial after giving off branches to flexor digitorum and hallucis longus tibialis posterior and popliteus (Tib). In a few experiments the nerves to the medial head of gastrocnemius (MG) and to soleus (Sol) were used separately while the one to the lateral head was omitted.

Recording The incoming volleys evoked by stimulation of peripheral nerves were recorded at the dorsal root entry zone usually in L7 Intracellular recordings from motoneurones (mostly PBSt and triceps) were made with glass capillary micro-electrodes (tip size 1 0 µm, impedance 2.5–6 Mohm) filled with M potassium citrate. Motoneurones were identified by their antidromic invasion from the muscle nerves Dorsal root potentials were recorded with bipolar electrodes one of the poles close to the cord and the other at the cut end of the filament (inter-electrode distance about 15 mm)

All signals were amplified filtered (LF filter Hz for intracellular and dorsal root recordings 1 Hz for dorsal root filament recordings) and displayed on a storage oscilloscope for visual inspection and simultaneously stored on a FM tape recorder (Sabre VI Sangamo) for later processing. The time course of long duration dorsal root potentials (DRPs) was distorted by the 1 Hz LF filter used, and appear shorter than they really were. For example as tested separately with DC recordings, a DRP with a duration of the negative wave of about 100 ms (1 Hz filter) corresponds to a real duration of some 200–.50 ms (DC), and a duration of the negative wa e of 1..5–150 ms (1 Hz filter) corresponds to a real duration of about 500 ms when recorded n DC mode Signals were usually computer averaged and then photographed from the oscilloscope screen

Stimulation Peripheral nerves were stimulated u bipolar hook electrodes The stimulu shocks were 0 1 ms square wa e pulses The strength of stimulation is given as multiples of the threshold (T) for the most excitable fibres in the nerves as determined from the incoming volleys at the appropriate dorsal root entry zone All conditioning-test series were performed with a low repetition frequency u ually around 1 Hz with long conditioning-test interval down to 0 5 Hz

Abbreviation DRP dorsal root potential EPSP excitatory postsynaptic potential. FRA flexor reflex afferent (cf Eccles & Lundberg 1959) IPSP inhibitory postsynaptic potential PSP post ynaptic potential RBSP rubro-bulbospinal path (f Jenesk g & J han son 197?)

RESULTS

I *Cutane u inhibit ry action on Group II mu cle affe ent path ays*

The interaction between cutaneous afferent and Group II muscle afferent path to motoneurones

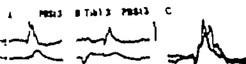


Fig 1A–C. Low threshold cutaneous inhibition of Group II-EPSP in GS motoneurone. Each record consists of 8 averaged sweeps, in A–B EPSPs in upper traces and incoming volley in lower traces. (A) Group II-EPSP evoked by 3T stimulation of PBSt nerve. (B) Same as A, but test EPSP preceded by low threshold stimulation of Tib nerve, 35 ms interval. (C) Central parts of A and B superimposed and enlarged to show suppression of late EPSP components (stippled area). Calibrations in B, horizontal bar 20 ms to A–B, vertical bar (positivity upwards, for upper traces) 3 mV to A–B.

d to primary afferent terminals was studied in conditioning-test experiments, using the PSPs and DRPs evoked by single shock stimulation of muscle nerves as tests. The results that emerged were rather complex and suggested that several mechanisms were involved in the interaction.

Fig. 1A–F is from a PBSt motoneurone and shows that a Group II-EPSP from the MG nerve appearing at around 2.5T (latency from end of Group I incoming volley 3.6 ms) was suppressed by a preceding (50 ms) volley in low threshold afferents either in the mixed Tib nerve (A) or in the purely cutaneous Su nerve (D). At longer intervals, illustrated in B and E with 260 ms, there was no longer a low threshold inhibition of this Group II EPSP. However when the strength of the conditioning stimulation was raised to 3T the suppressive effect re-appeared (C and F). The low threshold cutaneous suppressive effect did not seem to be an unspecific one, e.g. due to conductance changes in the motoneurone membrane, since in other cells, the Group II-PSP evoked from muscle afferents was suppressed by a preceding low threshold volley in cutaneous afferents even though this volley gave no appreciable PSP itself in the motoneurone under study as illustrated in Fig. A–C for a GS motoneurone. As detailed in Fig. 2C (stippled area), only the late PSP components were suppressed (see further Results section III). Hence the inhibitory effect on muscle afferent Group II-PSPs evoked by activity in low threshold cutaneous afferents is considered to affect the reflex path transmission rather than to be an effect on the motoneurones themselves.

A similar low threshold cutaneous suppression of transmission to primary afferent terminals is illustrated in Fig. 1G–I. In this case a supramaximal Group I stimulation (4T) of the PBSt nerve did not evoke any appreciable DRP in the S1 filament recorded from (G), but when the strength was raised to 5T (H) a considerable DRP developed. This Group II-DRP was heavily suppressed by a preceding conditioning volley in low threshold afferents of the Su nerve (I). Parallel to the recordings in Fig. 1G–I a MG motoneurone was investigated (not illustrated). In this cell a Group II EPSP appeared at slightly above 2.0T stimulation of the PBSt nerve and further increased on raising the strength from 4T to 5T. This EPSP was likewise suppressed by a conditioning volley in the Su nerve (1.4T, 30 ms interval).

The time course of low and high threshold cutaneous inhibitory actions on a Group II-IPSP is shown in greater detail in Fig. 3 for a Sol motoneurone. A and B show specimen records with different conditioning-test intervals of unconditioned Group II-IPSPs from the DP nerve (threshold for the IPSP in this cell was about 2.5T) in the lower traces and conditioned Group II-IPSPs in the upper traces. The full test series is illustrated in the diagram of Fig. 3C and demonstrates that with low threshold conditioning stimulation (SP 1.4T) the suppressive effect was maximal around 30 ms interval and then gradually faded off at 200–300 ms. With stronger conditioning stimulation (SP 3T) the suppression was more effective at all intervals where SP 1.4T also had an effect, but the inhibition was now longer lasting. A similar effect on the DRP simultaneously recorded in a caudal L6 dorsal root filament (most likely Group II-evoked since it appeared with single shock stimulation only well above 2T) is shown in Fig. 4. For the transmission to primary afferent terminals as well the high threshold conditioning stimulation (SP 3T) had a suppressive effect that was greater than and also outlasted the one obtained with the low threshold conditioning stimulation (SP 1.4T).

The more efficient and longer lasting inhibitory effect of high threshold conditioning stimulation was most probably not only a quantitative difference as indicated by the results presented in Fig. 5 with data from the same cell and from the same dorsal root filament as in Figs 3 and 4. In the diagram of Fig. 5 the transmission through the Group II pathways to the motoneurone (circles) and to the primary afferent terminals (triangles) has

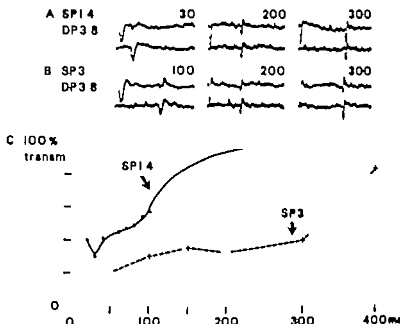


Fig 3 Time course of inhibition of Group II IPSP in a Sol motoneurone by low and high threshold conditioning stimulation of SP nerve (A) Specimen records (4 averaged) of unconditioned IPSPs (lower) and conditioned IPSPs (upper) at indicated conditioning-test intervals (ms) with low threshold cutaneous stimulation. (B) Same as A but with high threshold cutaneous stimulation. (C) Time course of suppression of transmission through Group II pathway to motoneurone Abscissa is interval between arrival of conditioning and test volleys at the spinal cord. Ordinate is transmission in percent of unconditioned value Filled circles and full line SP 1 4T crosses and interrupted line SP 3 T Positivity upwards in PSP traces.

been plotted against the strength of the conditioning stimulation of the SP nerve Two intervals were chosen one short (30 ms filled symbols) where low threshold conditioning was effective and one long (300 ms open symbols) where low threshold conditioning was no longer effective (cf Jeneskog & Johansson 1977 and Figs 1 3–4) As seen from the diagram the transmission of Group II effects was suppressed already at 1 1T conditioning stimulation of the SP nerve at the short interval and the suppression was more efficient at 1 2–1 4T At 300 ms interval on the other hand there was no inhibitory effect by the conditioning cutaneous volley until the strength of stimulation was raised to 1 6–1 8T Then the effectiveness of the conditioning stimulation grew with increased strength at least up to 3T the strongest conditioning stimulation used

These data should be correlated with those of Fig 6 where some features of the dorsal root potentials evoked by these same conditioning SP volleys themselves are shown. Specimen records of the DRPs evoked in a rostral S1 dorsal root filament at different stimulation strengths are shown in A In B is illustrated the DRPs at 1 6T and 1 4T stimulation and in the lowermost trace the difference between the two indicating that a new late component had appeared (component II DRP cf Carpenter et al 1963*a*) This component II had a latency of 15–20 ms from the incoming volley in contrast to the component I DRP which had a segmental latency of only 2 5 ms and a threshold of less than 1 1T In the diagram of Fig 6C the two curves show the amplitudes of the DRPs plotted against the stimulation strength Two intervals from the arrival of the incoming volley were chosen Firstly 15 ms, i e when the component II DRP (at higher strengths) had not yet started and secondly 35 ms, i e approximately at the maximal amplitude of the component II DRP As seen in the diagram there was no appreciable difference in amplitude between the curves at stimulation strengths up to 1 4T However from 1 6T and upwards the amplitude of the DRP at 35 ms interval suddenly was consistently greater than that at 15 ms interval thus indicating the presence at those strengths of the component II DRP

The appearance of the component II DRP at 1 6T stimulation correlates well with the appearance of

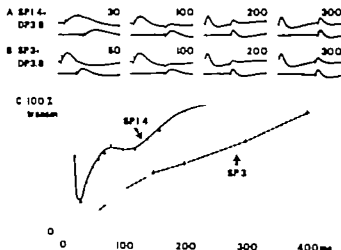


Fig 4 Time course of inhibition of Group II-DRP in caudal L6 dorsal root filament by low and high threshold conditioning stimulation of SP nerve. (A) Specimen records (4 averaged) of unconditioned DRPs (lower) and conditioned DRPs (upper) at indicated conditioning-test intervals (ms) with low threshold cutaneous stimulation. (B) Same as A, but with high threshold cutaneous stimulation (C) Time course of suppression of transmission through Group II pathway to primary afferent terminals Abscissa is interval between arrival of conditioning and test volley at the spinal cord. Ordinate is transmission in percent of unconditioned value Filled circles and full line SP 1.4T crosses and interrupted line SP 3T Negativity upwards in DRP traces.

the high threshold cutaneous inhibition of transmission through muscle afferent pathways at long intervals, as described in Fig. 5 These results indicate that more than one mechanism of inhibitory interaction between cutaneous afferent and muscle afferent pathways may be involved.

The inhibitory interaction patterns produced in other cases by conditioning stimulations applied to low and/or high threshold afferents in the Su or Tib nerves on Group II-evoked EPSPs, IPSPs or DRPs were similar to those presented in Figs 3 and 4

It should be stressed that the inhibitory interaction described was effective irrespective of whether or not the cutaneous conditioning volley evoked a PSP by itself in the motoneurone investigated, cf Fig. 2. The low threshold cutaneous inhibition of Group II-PSPs was studied in 23 tests in 14 flexor and extensor motoneurones. The latencies of those Group II-PSPs were 3.2–5.5 ms, as measured in seven cells from the end of the Group I incoming volley to the start of the PSP 4 of these 14 cells in addition showed Group II-PSPs with a shorter latency Such PSPs were, however not inhibited by cutaneous conditioning stimulation at the conditioning test intervals tested (see section III). The term low threshold cutaneous stimulation as used here was 1 1–1.5T for either nerve, except in one single case where the Su nerve was stimulated with 1 6 T This stimulation did not, however evoke a component II DRP in the caudal L6 dorsal root filament recorded from.

II *Cutaneous inhibitory action on a Group III muscle afferent pathway*

Group III PSPs (appearing around 10T stimulation, or above cf Eccles & Lundberg, 1959) were also inhibited by conditioning stimulation of cutaneous afferents with a time course similar to the one for Group II PSPs. Low threshold (up to 1.5T) cutaneous shocks were effective as conditioning stimuli in 13 tests in 10 flexor and extensor motoneurones. In two of these cells the conditioning cutaneous volley did not evoke a PSP by itself with the strength of stimulation effective for suppression of transmission through the Group III pathway In accordance with the observations for the Group II pathways. Examples of inhibition of Group III PSPs are shown in Fig. 7 The test Group III IPSP (22T) in a GS motoneurone (A) was suppressed by a preceding (35 ms) volley in low threshold afferents of the

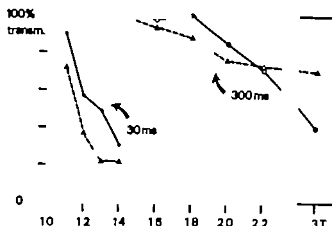


Fig 5 Transmission through Group II muscle afferent (DP 3 8T) pathways to a Sol motoneurone (circles and full lines) and to primary afferent terminals recorded as the DRP in a caudal L6 dorsal root filament (triangles and interrupted lines) plotted against the strength of conditioning cutaneous stimulation (SP nerve) Intervals between arrival of conditioning and test volleys at the spinal cord were 30 ms (filled symbols) and 300 ms (open symbols) Note that the abscissa is broken

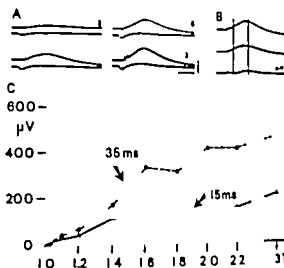


Fig 6 Amplitudes of rostral S1 dorsal root potentials evoked by graded stimulation of SP nerve. (A) Specimen records (4 averaged) of DRPs evoked by stimulation of SP nerve at indicated strengths (times T) in upper traces incoming volleys at the L7 dorsal root entry zone in lower traces. (B) Uppermost and middle traces DRPs by SP 1 6T and 1 4T stimulation, respectively Lowermost trace the computed difference between the two Observe the long latency for appearance of the high threshold component Vertical lines indicate 15 ms and 35 ms interval from arrival of incoming volleys, respectively (C) Amplitude of DRP at two different intervals from arrival of incoming volley at the spinal cord plotted against the strength of SP stimulation Note that the abscissa is broken. Filled triangles and full line 15 ms filled circles and interrupted line 35 ms. Calibrations in lower right A, horizontal bar 20 ms to A–B vertical bar (negativity upwards, only for DRPs) 0.5 mV to A–B

Tib nerve (B) Fig. 7C–D are from a MG motoneurone and show that the Group III IPSP (30T late component) was inhibited by a preceding (30 ms) volley in low threshold afferents also of the purely cutaneous Su nerve

In other cases (23 tests in 14 flexor and extensor motoneurones) a low threshold cutaneous conditioning stimulation (below 1 7T) did not affect the transmission through the Group III pathway to motoneurones Only when the strength of the conditioning stimulation was raised to or above 1 8T a suppressive effect could be demonstrated as exemplified in Fig 7E–H from another GS motoneurone The Group III IPSP evoked by a 50T ABSm volley (late component) was uninfluenced by a preceding (30 ms) conditioning Su volley with strengths up to 1 8T (not illustrated) However when the strength of the Su volley was raised to 2.0T the suppressive effect appeared as seen in some of the superimposed sweeps of Fig 7F With stronger conditioning shocks (G–H) the inhibition of the Group III IPSP became more efficient The results described indicate that with inhibition of transmission through Group III muscle afferent pathways too more than one mechanism is involved

In cells where long latency Group II as well as Group III PSPs were evoked by the test stimulation a low threshold cutaneous conditioning stimulation (up to 1 5T) either affected both muscle afferent PSP-components or none of them Likewise when the conditioning stimulation was in the high threshold range (at or above 1 8T for either cutaneous nerve used) the muscle afferent PSP components were either suppressed together or occasionally neither of them was influenced There was thus a parallelism in the sense that no matter the low or the high threshold cutaneous pathway was operating long latency Group II and Group III muscle afferent pathways were affected together

III *Further interactions between cutaneous and muscle afferent pathways to motoneurones*

As described above (section I) only Group II PSPs with rather long latencies (3.–5.5 ms) were suppressed by conditioning volleys in low and/or high threshold cutaneous afferents. In addition there

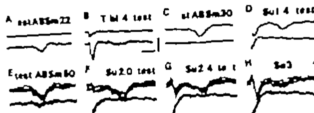


Fig. 7. Low and high threshold cutaneous inhibition of Group III-PSPs in motoneurones. (A–B) GS motoneurone with test IPSP from ABSm, and suppression of test IPSP by preceding (35 ms) volley in low threshold afferents of the Tib nerve (C–D) MG motoneurone with test IPSP from ABSm, and suppression of test IPSP by preceding (30 ms) olley in low threshold afferents of the Su nerve (E–H) GS motoneurone with test IPSP from ABSm, and suppression of test IPSP by preceding (30 ms) olley in high threshold afferents of the Su nerve. Observe linear effect in F. Averaged (8) eeps in A–B and C–D. Superimposed traces in E–H. PSPs in lower traces of A–B in upper traces of C–H. Other traces are incoming olleys at L7 dorsal root entry zone. Calibrations in B horizontal bar 20 ms to A–H vertical bar (positivity upwards, only for PSPs) 4 mV to A–B and E–H 8 mV to C–D.

were in some (=9) motoneurones also shorter latency EPSPs (latencies 2.0–3.1 ms, as measured in those nine cells from the end of the Group I incoming volley to the start of the EPSP), which were not suppressed by cutaneous conditioning volleys in the range of intervals tested.

The short and long latency Group II PSPs could be evoked in the same cell as shown for a PBSt motoneurone in Fig. 8 A–F. The EPSP appeared at a strength slightly above 2T stimulation of the GS nerve, and was of considerable size at 5T (A). The short latency component (2.0 ms) had the lowest threshold, but the long latency component (5.2 ms) was also evident at 5T. This component grew with stronger shocks to the GS nerve and was as pronounced with 6–7T (not illustrated) as with 10T stimulation (B) while the short latency component was probably maximal already at 5T (B compared to A). Hence the second component is considered also as a Group II muscle afferent EPSP. In this cell the long latency Group II EPSP was unaffected by the conditioning low threshold shocks to the cutaneous nerves tested (C–E). However the short latency Group II-EPSP was instead facilitated by the same conditioning shocks, whether (C) or not (D, E) these evoked PSPs by themselves in the motoneurone. If the conditioning-test sequence was reversed, as in Fig. 8F the cutaneous low threshold EPSP (lower trace) was facilitated (upper trace) by a conditioning olley in Group II (5T) afferents of the GS nerve. Such results further indicate that short and long latency Group II muscle afferent PSPs are evoked through different inter neuronal pathways.

All the three PSP components dealt with in the present investigation could occasionally be demonstrated in the same cell, as illustrated in Fig. 8 G–H from another PBSt motoneurone. With 10T stimulation of the homonymous PBSt nerve, the cell showed (G) the antidromic spike the short latency Group II-EPSP (at first arrow 3.0 ms), the long latency Group II-EPSP (at second arrow 4.1 ms) and the Group III-EPSP (at third arrow). The two Group II-PSPs both had a threshold around 2.5T. Below this strength only the antidromic spike was elicited. The third component did not appear until the strength was raised to 8–10T and was thus considered as a Group III-EPSP. This last component (but not the two first ones) was also even more pronounced with 15T stimulation. When, as in Fig. 8H (upper trace) the 10T PBSt shock was preceded (30 ms) by a low threshold volley in the Tib nerve the long latency Group II and the Group III EPSPs were suppressed simultaneously but the conditioning shock left the short latency Group II EPSP unaffected (compare Fig. 8H lower trace). It was even occasionally observed in other cells that, when both Group II PSP components were evoked by the test volley the long latency PSP was suppressed, while the short latency PSP was instead simultaneously facilitated.

Fig. 8 I–K are from a MG motoneurone with a test Group II-EPSP evoked by stimulation of the PBSt nerve. When this test EPSP was preceded (30

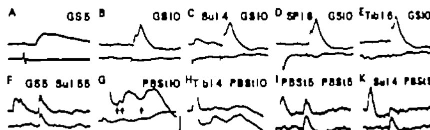


Fig 8 Further interactions between cutaneous and muscle afferent pathways to motoneurones (A–F) Spatial facilitation between low threshold cutaneous afferent and short latency Group II muscle afferent path to a PBSt motoneurone (A B) Group II EPSPs (see text) evoked by stimulation of GS nerve (C E) Facilitation of short latency component of Group II EPSP by preceding (35 ms) volleys in low threshold afferents of Su SP and Tib nerves respectively (F) Reversed sequence, lower trace test EPSP by stimulation of Su nerve (1.55T) upper trace test EPSP conditioned by preceding (30 ms) volley in Group II afferents of the GS nerve. (G–H) From a PBSt motoneurone (G) Antidromic spike and PSPs evoked by stimulation of PBSt nerve at 10T Observe 3 components marked by arrows (H) Lower trace, same as G but lower amplification upper trace same PSPs conditioned by preceding (30 ms) volley in Tib nerve at 1 4T Note that only second and third components are suppressed (I K) From a MG motoneurone Lower traces show test Group II EPSPs by PBSt 5T stimulation. Upper trace in (I) shows absence of interaction by preceding (30 ms) volley in the same afferents Upper trace in (K) shows low threshold cutaneous suppression of test Group II EPSP Lower traces in A E and G are incoming volleys at the L7 dorsal root entry zone Other traces are PSPs All traces are averaged sweeps 4 in A F I and K 8 in B–E and G–H Calibrations in G horizontal bar 4 ms to A G–H 20 ms to B–F and I K Vertical bar (positivity upward only for PSPs) 4 mV to A–G I–K 8 mV to H

ms) by a conditioning volley in the Su nerve (1 4T) there was the usual suppressive effect (K) but when the conditioning volley was in the same afferents as the test volley (I) there was no interaction at the same interval Such results were observed for a number of tests in different cells and indicate that the inhibition of transmission through the long latency Group II pathway (and also the Group III pathway) by low threshold cutaneous conditioning volleys did not depend upon a mechanism of post excitatory depression through the test pathway to the motoneurone which might have been the case if these low threshold cutaneous afferents somewhere shared interneurones with the test muscle afferent pathways

DISCUSSION

The primary aim of the present investigation was to gain further insight into the mechanisms responsible for the suppression of transmission through Groups II and III muscle afferent segmental pathways by activity in cutaneous afferents which was described by Jeneskog & Johansson (1977) Similar interactions between cutaneous and muscle afferent pathways to motoneurones were early studied by Kuno & Perl (1960) They showed a suppression of Groups II and III effects on the excitability in motor nuclei by near maximal sural volley (for myelinated fibres)" (Kuno & Perl 1960 p 11) and thus it is not possible to decide whether the effective afferents were low or high threshold ones as those terms are used here Later Eccles et al (196 b) studied inhibitory interactions between cutaneous and muscle afferent pathways in a number of test situations They found i a that the reflex discharge in flexor muscle nerves as well as the dorsal root potentials evoked by the same type of test volleys were inhibited following single conditioning volleys in cutaneous afferents The standard stimulation strength they used for cutaneous shocks was 4T (cf Eccles et al 196 a) thus again including both low and high threshold afferents They concluded that the inhibitory interaction was due to a presynaptic mechanism working between different FRA systems (flexor reflex afferents Eccles & Lundberg 1959) at the segmental level of the spinal cord They also suggested (Eccles et al 196 b p 77) that the interaction described by Kuno & Perl (1960) was another example of presynaptic inhibition within the FRA systems challenging the original interpretation Contralateral cutaneous volleys have also been shown to inhibit the transmission in

spinal FRA pathways (Eccles et al. 1964) by a presumed presynaptic mechanism. These authors also used strong conditioning shocks, but an appreciable part of the inhibition was produced by other low threshold afferents (up to 1.7–1.8T Eccles et al. 1964, their Fig. 5). Some evidence also exists to the effect that identified Group II muscle afferents are recipients of presynaptic inhibition from cutaneous afferents (Eccles et al. 1963). Unfortunately only strong (4T) stimulation of cutaneous nerves was used to produce the primary afferent depolarization in Group II muscle afferents too.

Taken together, the inhibition of transmission in high threshold cutaneous and muscle afferent (FRA) pathways to motoneurones and to primary afferent terminals has been attributed "in whole or in part" (Schmidt 1973) to a presynaptic mechanism.

The present experiments were performed on acute spinal cats, in order to exclude possible long spinal or supraspinal reflex loops (e.g. Shimamura et al. 1976). As low threshold cutaneous afferents only with distal receptive fields are effective in suppressing the transmission through high threshold muscle afferent pathways (Jenekog & Johansson 1977), the sural, the cutaneous part of the superficial peroneal and the tibial nerves were used for conditioning stimulation. Concerning the tibial nerve, which also contains joint and muscle afferents, cutaneous afferents were assumed to be responsible for the suppressive effects because the threshold range and the time course of effects were the same as for conditioning with afferents in the purely cutaneous nerves. Furthermore, the effects by tibial nerve stimulation appeared in parallel to the cutaneous cord dorsum potential as recorded by the surface electrode at the dorsal root entry zone. A contribution to the inhibitory effects from tibial nerve stimulation by Renshaw cell activation elicited via antidromic stimulation of motor axons seems unlikely since a recent anatomical investigation has shown that tibial motoneurones do not give off recurrent collaterals at all (Cullheim & Kellerth 1978). Finally similar suppressive effects have been obtained with electrical stimulation within the pad (Jenekog & Johansson 1977), clearly suggesting that cutaneous afferents in the tibial nerve may produce this kind of inhibitory interaction.

The latencies presented for Group II-PSPs were measured from the end of the Group I incoming volley at the dorsal root entry zone to the foot of the PSP and are thus not true "central latencies". They were however only used to differentiate between Group II PSPs which were suppressed by cutaneous conditioning stimulation ("long latency"—presumed polysynaptic) and those which were not suppressed, but might instead be facilitated ("short latency"—presumed disynaptic for EPSPs).

The present investigation demonstrated that the inhibitory interaction between cutaneous and Groups II and III muscle afferent pathways seems to involve more than one mechanism. As far as the transmission to motoneurones is concerned, the inhibitory interactions were evident whether or not the cutaneous volley evoked a PSP by itself in the motoneurone under study. Hence it is unlikely that the suppressive effects were dependent upon action on the motoneurone membrane. It is concluded that the inhibitory actions were exerted at stages in the reflex path prior to the motoneurone itself. This conclusion is supported by the fact that the short latency excitatory Group II pathway to flexor and extensor motoneurones was never observed to be suppressed by the cutaneous conditioning volleys. Furthermore, excitatory and inhibitory polysynaptic pathways to motoneurones were similarly affected, and an involvement of Renshaw cells is thus considered unlikely, as they seem to influence only the transmission through pathways involving the reciprocal Ia inhibitory interneurones (Hultborn et al. 1971).

The transmission through high threshold muscle afferent polysynaptic pathways was suppressed by one path activated from low threshold cutaneous afferents. Pathways affected were Group II polysynaptic to alpha-motoneurones and to primary afferent terminals as well as Group III paths to alpha-motoneurones. The inhibitory effect was produced by cutaneous afferents also in the very low threshold (<1.1T) range. It was not possible to delineate the highest threshold cutaneous afferents involved, because with conditioning shocks of 1.6–1.8T or greater another inhibitory path was also put into action. This high threshold cutaneous path also affected all reflex paths studied, but its characteristics were different, e.g. the time course of its action was slower than that of the low threshold path.

For a number of reasons it is suggested that the difference in time course of the inhibition of transmission through the polysynaptic muscle afferent pathways by low and high threshold cutaneous afferents is not merely due to a quantitative differ-

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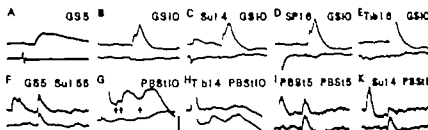


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For a number of reasons it is suggested that the difference in time course of the inhibition of transmission through the polysynaptic muscle afferent pathways by low and high threshold cutaneous afferents is not merely due to a quantitative differ

ence Firstly the low threshold path was rather labile and in a few experiments only the high threshold path could be studied because suppression of transmission was not evident until the strength of the conditioning cutaneous stimulation was raised above 1 6–2 0T In other experiments the transmission to some motoneurones was affected by the low as well as the high threshold cutaneous path while for the transmission to other motoneurones only the high threshold path was operating Secondly when investigating the inhibitory interaction at long conditioning test intervals (e g 300 ms as in Fig 5) it was found that the lowest threshold for a suppressive effect was 1 6–1 8T Thirdly with high threshold stimulation all three cutaneous nerves were effective in suppressing the reflex transmission while with low threshold conditioning shocks commonly only one or two of the cutaneous nerves were effective

Regarding the mechanisms of inhibition of transmission through the muscle afferent reflex pathways the two cutaneous paths will be discussed separately Concerning the high threshold path the threshold for its activation was the same as that for evoking a component II DRP The component II DRP evoked by stimulation of cutaneous nerves is considered to be an "FRA effect from cutaneous afferents (Carpenter et al 1963*a*) and is presumed to represent primary afferent depolarization in FRA terminals Taking into account also the large peripheral receptive field for activation of this cutaneous path (all three cutaneous nerves equally effective) it is thus suggested in agreement with earlier studies (see Schmidt 1973) that *the inhibition of transmission through polysynaptic Group II and III muscle afferent pathways by activity in high threshold (above 1 5T) cutaneous afferents is due to presynaptic inhibition between these primary afferent systems* The apparent disagreement between the duration of the DRPs presented in the figures and the duration of inhibition of reflex path transmission attributed to the same mechanism is explained in Methods section It is however not excluded that postsynaptic interneuronal inhibition may also contribute to this interaction

The low threshold cutaneous inhibition of transmission through the reflex pathways cannot how ever be explained by the same mechanism although the time course of this inhibitory interaction is also rather long. It is produced by cutaneous afferents which do not evoke a component II DRP but only a component I DRP This DRP (evoked by the low threshold cutaneous afferents, and known to give primary afferent depolarization in other cutaneous afferents) cannot be considered as part of the FRA effect from cutaneous afferents (cf Carpenter et al 1963*a*) This is further indicated by the fact that in the decerebrate cat the inhibition of reflex paths (from Groups I*b* II and III muscle afferents) by the dorsal reticulospinal system does not include the path evoking the component I DRP (Carpenter et al 1963*a* Engberg et al 1968*a*) There are furthermore a number of ascending paths that are activated from the FRA (Oscarsson 1973) With regard to the contribution from cutaneous afferents to the activation of ventral spino-olivocerebellar paths the stimulation strength must be 1 5–3 0T or above (Oscarsson & Sjölund 1977), excluding the low threshold ones from the FRA concept at least in this respect

Altogether and taking into account also the restricted peripheral receptive field for the low threshold cutaneous afferents responsible (only nerves with distal fields, and commonly only one of them) for the inhibition of transmission through the high threshold muscle afferent pathways, it is unlikely that this cutaneous path belongs to the FRA as originally defined (Eccles & Lundberg 1959).

There is still the possibility that the low threshold cutaneous path might inhibit the reflex transmission by another presynaptic mechanism, separated from the FRA mechanism There is, however no available evidence suggesting that the component I DRP represents primary afferent depolarization in terminals other than those of cutaneous afferents (Carpenter et al 1963*a*) In fact, only the rather long time course of the low threshold cutaneous inhibitory action reminds of a presynaptic action It is however well known that postsynaptic interneuronal inhibition may have a rather long time course in some systems The dorsal reticulospinal system (Engberg et al 1968*a* *b*) or the RBSI (Jeneskog & Johansson 1977) is one example where postsynaptic inhibition of transmission through reflex pathways has a long time course The latter authors described furthermore a similar inhibitory action from low threshold cutaneous afferents with distal receptive fields on the same reflex pathways The effect was suggested to be a postsynaptic inhibition at an interneuronal level because facilitatory interaction between descending and segmental actions was revealed

There are several reasons to belie e that the low threshold cutaneous inhibition studied by Jeneskog & Johansson (1977) and the one studied in the present investigation is the same mechanism. The time course of effects is similar the peripheral receptive field for the cutaneous afferents in question is similarly restricted and the range of thresholds for their activation is the same. The most likely explanation at present, for the mechanism is therefore that *low threshold (less than 1.5T) cutaneous afferents may inhibit the transmission through polysynaptic Groups II and III muscle aff re t pathways at an interneuronal le el.*

Alternatively the low threshold cutaneous inhibition could be an "experimental artifact" due to a postexcitatory depression in Groups II and III muscle afferent pathways, provided that the low threshold cutaneous afferents also activated interneurones in these paths. This explanation is however not likely because when conditioning and test volleys were applied to the same afferents and thus should have affected the same interneuronal system, there was no inhibitory effect on the transmission through the test reflex pathway but with low threshold cutaneous conditioning the suppression as readily apparent (cf Fig 8 I–K)

It should finally be recalled that regarding Group II muscle afferent pathways, only the polysynaptic ones were affected by the two mechanisms described above. The short latency Group II path to flexor motoneurones which is considered to be disynaptic (Lundberg et al. 1975), was never inhibited by preceding activity in cutaneous afferents. On the other hand, it was sometimes possible to demonstrate a facilitatory interaction between low threshold cutaneous afferents and (low threshold) Group II muscle afferents in the transmission to motoneurones, regardless of whether the cutaneous or the muscle afferent olley was used to elicit the test response If this Group II muscle afferent pathway is disynaptic, the facilitatory effect from low threshold cutaneous afferents would be exerted at the only interneurone involved as the facilitation could be produced by cutaneous nerve stimulation that did not evoke PSP the motoneurone by itself. The influence from cutaneous afferents is thus assumed to be disynaptic on the Group II interneurone, as the shortest path to motoneurones from low threshold cutaneous afferents is presumably trisynaptic (Lundberg 1975). Lundberg et al (1977) ha e recently reported a similar convergence from cutaneous joint and Group II muscle afferents in inhibitory reflex pathways to extensor motoneurones Such a convergence between low threshold cutaneous and Group II muscle afferents (most likely from spindle secondary endings) in reflex paths is of great interest, as also recently pointed out by Appelberg et al (1977). These authors reported dynamic fusimotor neurones to be strongly influenced by acti ity in such afferents. From the point of iew of load compensation in motor control a co-operation of the afferents in question on skeletomotor as well as on dynamic fusimotor neurones would seem to be of a particular value

The author wishes to thank Doc. B. Appelberg for helpful discussions and valuable criticism throughout this work and Mrs G. Kristrom for skillful technical assistance. This work was supported by the Swedish Medical Research Council (Project No B77-04X-04957-01).

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Acta Physiol Scand 1979 107 309–312

Morphological and mechanical properties of small mesenteric arteries and veins in spontaneously hypertensive rats

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AALKJÆR, C & MULVANY M. J Morphological and mechanical properties of small mesenteric arteries and veins in spontaneously hypertensive rats. Acta Physiol Scand 1979 107 309–312. Received 2 Feb 1979 ISSN 0001-6772. Institute of Biophysics, University of Aarhus Denmark.

Segments of small mesenteric arteries (~150 μm lumen diameter) and of corresponding veins were taken from 5-month-old spontaneously hypertensive rats (SHR) and from age matched Wistar Kyoto (WKY) controls. The segments were mounted on a myograph which enabled their mechanical and morphological parameters to be investigated simultaneously. Compared with the WKY arteries the lumen diameter of the SHR arteries was smaller while the media thickness and active wall tension response were greater. On the other hand there were no differences between the corresponding veins from SHR and WKY animals although, compared with the arteries, the veins had a greater lumen diameter, a smaller media thickness and a smaller tension response. The findings suggest that the morphological and mechanical differences seen in arteries from SHR are not found on the venous side.

Key words: Morphology, arteries, veins, spontaneously hypertensive rats

Essential hypertension in man is accompanied by an increase in the wall thickness of resistance vessels, in particular those having lumen diameters of about 100 μm (Furuyama 1962, Suwa & Takahashi 1971). In the spontaneously hypertensive rat (SHR), widely used as a model for human essential hypertension (Udenfriend et al. 1975) we have demonstrated previously a greater wall thickness of arteries of diameter ~150 μm (Mulvany, Hansen & Aalkjær 1978). In smaller arteries and arterioles the media thickness is reported to be reduced (Bohlen & Lobach 1978, Henrich, Hertel & Assmann 1978), while in the portal vein wall hypertrophy has been observed (Greenberg & Bohr 1975). This has therefore raised the possibility that in the SHR wall (and media) hypertrophy is a general characteristic of the larger vessels, rather than being associated with the greater pressure to which the vessels are exposed as suggested by the indirect perfusion experiments of Folkow et al. (1974). To test this possibility we have therefore in the present study sought to determine whether the differences we have found in 150 μm arterial resistance vessels are also present in the corresponding veins.

MATERIAL AND METHODS

Segments of third generation vessels of a. or v. mesenterica sup. about 0.7 mm long, were taken from 5-month-old spontaneously hypertensive rats (SHR) and from age matched normotensive Wistar Kyoto (WKY) controls, all supplied by Møllegaards Avlslaboratorium, Ll Skensved, Denmark. In every second experiment it was the arterial vessel which was first dissected out and tested, and then later during the same day the corresponding venous vessel went through the same procedure. In the other experiments it was the venous vessel which was tested first.

The myograph used for testing the vessels has been described in detail earlier (Mulvany & Halpern 1977, Mulvany et al. 1978). With this arrangement we were able to measure simultaneously the internal circumference of the vessel, the wall and media thicknes as well as the vessel' isometric wall tension.

Nomenclature

The force developed by the vessel is expressed as circumferential wall tension, T, defined by:

$T = F/2a$

where F is the force measured by the force transducer and a is the segment length. The active wall tension

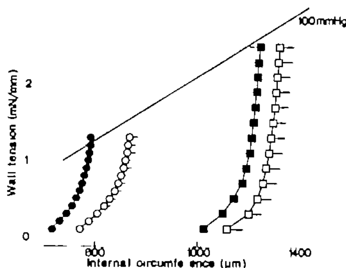


Fig 1 Relation of passive wall tension and internal circumference of arteries (circles) and veins (squares) from SHRs (filled symbols) and WKYs (open symbols) Each point shows the mean internal circumference for each wall tension level obtained from 9 expts (where necessary by interpolation) Horizontal bars show S E The line marked 100 mmHg joins points which are equivalent (see text) to an effective pressure of 100 mmHg (13 3 mN/mm²)

is the total wall tension following activation less the wall tension immediately before activation

To estimate the characteristics which the vessels would have had in situ we have calculated (Mulvany & Halpern 1977) the *effective lumen diameter* *d* and (using Laplace equation) the *effective pressure* *p* according to the following equations.

$d = L/\pi$
$p = T/(d/2)$

where L is the internal circumference

Solutions

The physiological salt solution (PSS) used had the following composition in mM (Murphy Herbby & Mergerman 1974) NaCl 119 $NaHCO_3$ 14 9 KCl 4 7 KH_2PO_4 1 18 $MgSO_4$ 1 17 $CaCl_2$ 1 6 ethylenediaminetetraacetic acid (EDTA) 0 026, glucose, 5.5 Vessels were activated by a potassium solution (K PSS) which was as for PSS but with an equimolar substitution of KCl for NaCl and with 2.5 mM CaCl Solutions were bubbled with 5% CO_2 in O_2 adjusted to pH 7 4 and held at 37°C

Protocol

The protocol used was in general similar to that described previously (Mulvany et al 1978). First with the vessels in PSS the internal circumference was increased in steps of about 20 µm at 1 min intervals until the effective pressure (see above) equalled 100 mmHg (13.3 mN/mm²) the internal circumference being defined as L_{100} Vessels were then released through a similar sequence and the passive characteristic was then taken as the curve defined by the points obtained in this sequence

Vessels were then set to an internal circumference $L_1 = 0\ 9L_{100}$ and mean wall and media thicknesses were estimated in two ways. First as described previously, the microscope was focussed on the outer edge of the mounting wires so that the wall (and the media within it) could be seen in longitudinal section and measured using an ocular micrometer Second as a new method, the microscope was focussed on the portion of the upper wall between the wires and the preparation was optically sectioned that is we measured the vertical movement of the microscope stage required to move the plane of focus through the successive layers of the wall The advantage of the second method was that we could make measurements on those portions of the wall which were not in contact with the mounting wires. Measurements were made in 12 positions with each method and the reported values are the means of these measurements

The active characteristics of vessels were then determined by activating them at L_1 with the high potassium solution (K PSS) and measuring the active wall tension after 3 min Finally vessels were fixed on the myograph at L_1 in 5% glutaraldehyde post-fixed in osmium tetroxide and embedded in Epon for later histological examination

RESULTS

The passive wall tension—internal circumference relations of the SHR and WKY arterial and venous

Table 1 *Morphological and mechanical parameters of the vessels used in this investigation*

Values show mean ± S E of parameters measured with vessel held at normalized internal circumference L_1

	Artery		Vein	
	SHR	WKY	SHR	WKY
Number of vessels	9	9	9	9
Effective lumen µm	16_ 0±5 7	214 0±5 4	366 0±12 7	377 0±18 7
Wall thickness µm	17 7±1 1	13.3±0 9	5 7± 0 2	6 8_ 0 4
Media thickness µm	12 6±0.5	8 5±0 5	4 2± 0 1	4 6± 0_
Active wall tension mN/mm	3 0±0_	_3±0 1	0 5± 0 1	0.5± 0 1

Indicate significant difference between the SHR parameter concerned and the corresponding WKY parameter at the 5% and 0 1% levels respectively as determined by the two-tail Student *t* test

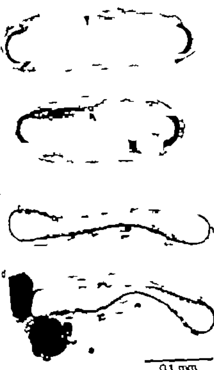


Fig 2 Cross-sections of essels fixed on the myograph at L_1 (a) WKY artery (b) SHR artery ( ) WKY vein (d) SHR vein. Sections stained with toluidene blue

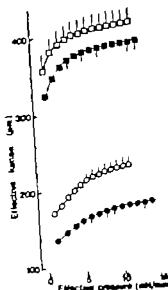


Fig 3 Relation between effective pressure and effecti lumen calculated as described in Methods from data used for Fig 1 Symbol as in Fig. 1

vessels are shown in Fig. 1 For any given wall tension the SHR arteries had a smaller internal circumference (L_0/π=16 µm) than the WKY arteries (L_0/π=214 µm) There was however no significant difference between the corresponding internal circumference of the SHR and WKY veins (L_0/π=366 µm and 377 µm respectively) both being about twice as large as those of the arteries. By contrast, with the essels set to internal cir cumference L_1, media thicknesses of the SHR arteries (12 6 µm) were greater than those of the WKY arteries (8 5 µm) while there was no significant difference between media thicknesses i the SHR and WKY veins (5 7 µm and 6.8 µm respectively). Similar findings were made as regards the wall thicknesses. These results are summarised in Table 1 Light micrographs of cross-sections of vessels fixed at L_1 (Fig.) indicated that while there were about 4 layers of smooth muscle cells in the SHR arteries or about 3 layers of cells in the WKY arteries the veins contained less than one layer That is the media of the veins was irregular and did not fully cover the internal elastic lamina.

The active response of the vessels to K PSS is show in Table I The acti e wall tension produced was greater in the SHR arteries (3 0 mN/mm) than in the WKY arteries (2.3 mN/mm). By contrast there was no difference between the active wall tensions produced by the SHR and WKY veins. The active wall tension of the veins (0.5 mN/mm) was howe er substantially lower than the active wall tension developed by the arteries.

DISCUSSION

The results of this invest gation show that the thickened media and decreased lumen which I seen n 150 µm arterial resistance vessels in the SHR mesenteric bed (Mulvany et al 1978) are structural differences which are not present in the corresponding veins Bohlen, Gore & Hutchins (1977) have show that the absolute blood pressure differences of SHR and WKY animals are confined almost entirely to the precapillary vessels. Therefore our finding that the structural differences are also confined to precapillary vessels suggests that there is a connection between these vascular differences

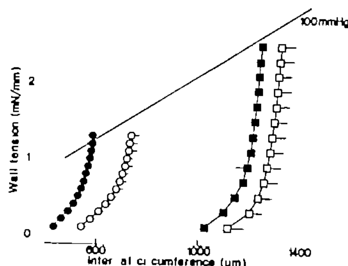


Fig 1 Relation of passive wall tension and internal circumference of arteries (circles) and veins (squares) from SHRs (filled symbols) and WKYs (open symbols). Each point shows the mean internal circumference for each wall tension level obtained from 9 expts (where necessary by interpolation). Horizontal bars show S E. The line marked 100 mmHg joins points which are equivalent (see text) to an effective pressure of 100 mmHg (13.3 mN/mm²)

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$d = L/\pi$
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where L is the internal circumference

Solutions

The physiological salt solution (PSS) used had the following composition in mM (Murphy Herlihy & Megerman 1974) NaCl 119 $NaHCO_3$ 14 9 KCl 4 7 KH_2PO_4 1 18 $MgSO_4$ 1 17 $CaCl_2$ 1 6 ethylenediaminetetraacetic acid (EDTA) 0 026 glucose, 5.5 Vessels were activated by a potassium solution (K PSS) which was as for PSS but with an equimolar substitution of KCl for NaCl and with 2.5 mM $CaCl_2$. Solutions were bubbled with 5% CO_2 in O_2 adjusted to pH 7 4 and held at 37°C

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Vessels were then set to an internal circumference $L_0 = 0\ 9L_{100}$, and mean wall and media thicknesses estimated in two ways. First as described previously the microscope was focussed on the outer edge of the mounting wires so that the wall (and the media within it) could be seen in longitudinal section and measured using an ocular micrometer. Second as a new method, the microscope was focussed on the portion of the upper wall between the wires and the preparation was optically sectioned that is we measured the vertical movement of the microscope stage required to move the plane of focus through the successive layers of the wall. The advantage of the second method was that we could make measurements on those portions of the wall which were not in contact with the mounting wires. Measurements were made in 1 positions with each method and the reported values are the means of these measurements

The active characteristics of vessels were then determined by activating them at L_0 with the high potassium solution (K PSS) and measuring the active wall tension after 3 min. Finally vessels were fixed on the myograph at L_0 in 2.5% glutaraldehyde post-fixed in osmium tetroxide and embedded in Epon for later histological examination

RESULTS

The passive wall tension—internal circumference relations of the SHR and WKY arterial and venous

Table I *Morphological and mechanical parameters of the vessels used in the investigation*
Values show mean ± S E of parameters measured with vessel held at normalized internal circumference L_0

	Artery		Vein	
	SHR	WKY	SHR	WKY
Number of vessels	9	9	9	9
Effective lumen µm	162 0 ± 5 7	140 ± 5 4	366 0 ± 1 7	377 0 ± 18 7
Wall thickness µm	17 7 ± 1 1	13 3 ± 0 9	5 7 ± 0	6.8 ± 0 4
Media thickness, µm	1 6 ± 0 5	8 5 ± 0 5	4 ± 0 1	4 6 ± 0
Active wall tension, mN/mm	3 0 ± 0 2	3 ± 0 1	0 5 ± 0 1	0.5 ± 0 1

Indicate significant difference between the SHR parameter concerned and the corresponding WKY parameter at the 5% and 0 1% levels respectively as determined by the two-tail Student *t* test

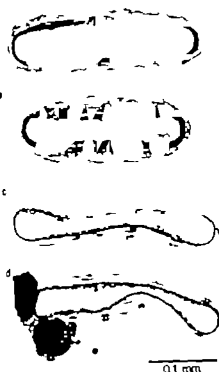


Fig 2 Cross-sections of essels fixed on the myograph L_{1} (*a*) WKY artery (*b*) SHR artery ( ) WKY vein, (*d*) SHR vein Sections stained with toluidene blue

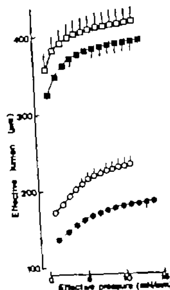


Fig 3 Relation between effective pressure and effecti lumen calculated as described in Methods from data used for Fig 1 Symbols as in F g. 1

vessels are show in Fig. 1 For any given wall tension the SHR arteries had a smaller internal circumference (L_{0}/π=162 μm) than the WKY arteries (L_{0}/π 14 μm). There was howe er no significant difference between the corresponding internal circumference of the SHR and WKY veins (L_{0}/π=366 μm and 377 μm, respectively), both being about t ice as large as those of the arteries By contrast, with the essels set to internal cir cumference L_{1}, media thicknesses of the SHR arteries (12.6 μm) ere greater than those of the WKY arteries (8.5 μm) while there was no significant difference bet een media thicknesses in the SHR and WKY eins (5 7 μm and 6.8 μm, respectively). Similar findings were made as regards the wall thicknesses. These results are summarised in Table 1. Light micrographs of cross-sections of vessels fixed at L_{1} (Fig. 2) indicated that while there were about 4 layers of smooth muscle cells in the SHR arteries, or about 3 layers of cells in the WKY arteries the eins contained less than one layer That is the media of the veins was irregular and did not fully cover the internal elastic lamina.

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and the loading to which they have been subjected Whether the structural differences are a result or a cause of the increased pressure cannot at present be determined We have however shown in a recent age study (Warshaw Mulvany & Halpern 1979) that the media hypertrophy occurs concurrently with the increase in blood pressure of the SHRs from the age of 6 weeks Thus in vessels of this size it seems at least that media thickness and transmural blood pressure are closely associated and that the structural differences seen are not general properties of the vasculature This conclusion is therefore in contrast to Greenberg & Bohr's (1975) finding that there is hypertrophy of the SHR portal vein in which there is little elevation of blood pressure One possible reason for the discrepancy is that Greenberg & Bohr's control animals were stock Wistar rats whereas ours were Wistar Kyoto rats

Hallbäck Lundgren & Weiss (1974) have from measurements of the perfusion pressure and flow rate in an isolated vascular bed determined the relation between the radius of an average vessel and its transmural pressure under relaxed conditions Fig. 3 shows the calculated effective pressure—effective lumen relations of our vessels based (as described in Methods) on the internal circumference—wall tension relations of the relaxed vessels (Fig 1) The results suggest that the distensibility of the SHR arteries is lower than that of the WKY arteries and also that the veins have a smaller distensibility than the arteries for transmural pressures above about 10 mN/mm² (75 mmHg) These findings are remarkably similar to those presented by Hallbäck et al (1974) The decreased distensibility of the arteries could be due either to a thicker media or to altered elastic properties of the wall components Our morphological measurements indicate that the decreased distensibility of the arteries is at least in part due to the increased media thickness. By contrast this cannot explain the lower distensibility of the veins for here the wall is much thinner Therefore in the veins it must be the elastic properties of the wall components which differ from those of the arteries

In conclusion our results support the indirect evidence of Folkow et al (1974) based on perfusion studies that the structural changes within the SHR vasculature are confined to the precapillary vessels with little change in the structure of the postcapillary vessels

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cta Physiol Scand 1979 107 313–318

between serum IgE levels and anaphylactic histamine release from isolated rat mast cells

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SYDBOM A. & KARLSSON T Relationship between serum IgE levels and anaphylactic histamine release from isolated rat mast cells Acta Physiol Scand 1979 107 313–318. Received 11 April 1979 ISSN 0001-6772 Department of Pharmacology Karolinska Institutet, Stockholm, Sweden, and Department of Medical and Physiological Chemistry Biomedical Centre Uppsala, Sweden

Inbred Hooded Lister rats were immunized with egg albumin with B. pertussis vaccine used as an adjuvant. The serum levels of total IgE and IgE antibody (egg albumin specific) were determined by radioimmunoassay techniques before and after immunization. The basic level of total IgE in serum was 560±110 ng/ml. After immunization maximal peak at day 11 of 1940±160 ng/ml was registered Anti-egg albumin IgE antibody showed maximum around day 13 of 75±11 units/ml. Pleural mast cells were isolated on Ficoll between day 14 and 20 after immunization. A significant negative correlation between the basic total IgE level and histamine release by antigen (egg albumin) was found and also significant positive correlation of specific IgE antibody (determined at day 11) and histamine release. The correlation between IgE level and histamine release was slightly improved if instead the ratio of specific IgE antibody over total IgE was used

Key words inbred Hooded Lister rats, immunization, pertussis vaccine, rat IgE-radioimmunoassay

IgE antibody is considered to be responsible for the anaphylactic reactions of antigen exposed sensitized mast cells of many species among them the rat (Stechschulte et al. 1970) There have been a number of reports attempting to correlate the serum IgE levels with the clinical symptoms (see e.g. Elgefors et al. 1974) The results are diverging and the significance of the level of circulating IgE for the development of atopic reactions has been disputed (Hendersen et al 1971)

By the use of animal models for immunization studies it has been possible to follow the IgE antibody response by passive cutaneous anaphylaxis (PCA) or the histamine release from mast cells from immunized animals. With PCA it is however often difficult to get well reproducible results. Jarrett & Stewart (1973) reported that in the rat there is no direct relationship between the size of skin test reactions and the level of specific circulating IgE antibody tested by PCA. Neither did Yamamoto et al. (1974) register any correlation between the reagin titre and histamine release from different in vitro systems in the rat. However Stahl Skov et al. (1977) have measured histamine release from human basophils and found a significant correlation between specific but not total IgE and cell sensitivity when IgE had been determined by a radioimmunoassay technique (RAST) After the discovery of IgE myelomas in LOU rats (Bazin et al. 1973 1974) new techniques to measure total IgE and specific IgE antibody in rats have been developed. Radioimmunoassay techniques used for measurement of human IgE can be used also for rat IgE. Assays based on paper disc coupled anti IgE antibodies (PRIST paper radio immunosorbent test) for total IgE (Karlsson et al 1979) and antigens (allergens) (PRAST-paper radio allergo sorbent test) for specific IgE antibody have been described (Karlsson et al 1979*b*). These techniques have made it possible to detect low levels as well as small differences in total IgE and specific IgE antibody levels down to ng amounts.

and the loading to which they have been subjected Whether the structural differences are a result or a cause of the increased pressure cannot at present be determined We have however shown in a recent age study (Warshaw Mulvany & Halpern 1979) that the media hypertrophy occurs concurrently with the increase in blood pressure of the SHRs from the age of 6 weeks Thus in vessels of this size it seems at least that media thickness and transmural blood pressure are closely associated and that the structural differences seen are not general properties of the vasculature This conclusion is therefore in contrast to Greenberg & Bohr's (1975) finding that there is hypertrophy of the SHR portal vein in which there is little elevation of blood pressure One possible reason for the discrepancy is that Greenberg & Bohr's control animals were stock Wistar rats whereas ours were Wistar Kyoto rats

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Table 1. *Concentration of IgE in rat serum*

Total IgE and specific IgE antibody was determined in serum samples taken from Hooded Lister rat just prior to and at different day after immunization with 20 μg egg albumin in pertussis vaccine

Days after immunization		Specific IgE antibody (units/ml) $\bar{x}$ ± S.E.			Total IgE (ng/ml) $\bar{x}$ ± S.E.	
Basic					560±110	13
5		6± 2	1	NS	630± 70	26
11		39± 5	32		1 940±160	37
13		80±12	5		1 50±140	4
14	NS	70±19	5		680±130	3
19	NS	65± 9	10		630±140	10
20	NS	63± 7	7		1 000±130	7
21		49± 5	6		910±140	6
27		47± 7	5	NS	780_ 90	7

Student' t test between specific IgE antibody on day 13 and subsequent day
Student' t-test between basic total IgE and total IgE on day 5 and day 27 resp
Student' t-test between total IgE on day 11 and subsequent day
NS not significant 0 01<p<0 05 ** 0.001<0.01 *** p<0 001

RESULTS

The serum IgE patt rn after immunization

Blood samples for IgE determinations were taken maximally 4 times from one single rat before immunization, at day 5 day 11 and finally when the rat was killed between day 12 and day 27 The basic total IgE level for these Hooded Lister rats were 560±110 ng/ml (n=13) Before day 5 no changes could be seen but between day 5 and 11 both the total IgE and the IgE antibody levels were increased. Between day 13 and 14 there was a maximum of IgE antibody of about 75±11 units/ml (n 10) (Fig. 1 Table 1) The specific IgE antibody level does not decrease significantly until day 21 The total IgE shows maximum around day 11 of about 1940±160 g/ml (n=37) The total serum IgE declines faster than the specific IgE antibody There is an apparent increase in total IgE at day 20. However when examining the data more carefully we could see that those rats with high levels on day 20 also showed extremely high IgE levels on day 11

Correlation betw en IgE levels nd histamine leas

Of totally 48 rats used only 22 rats were used for histamine release studies and tested for all parameters mentioned, namely basic total IgE and total IgE and specific IgE antibody on day 11 as well as on the day of death The results from these 22 rats were used for correlation calculations

Basi total IgE Histamine release was measured after challenge of isolated sensitized mast cells with antigen The antigen concentration 10 μg/ml was the lowest concentration that gave maximal response and released 18 4±1.2% (n=51) of histamine from mast cells isolated from immunized Hooded Lister rats. A significant negative correlation could be seen between the per cent histamine released and the basic total IgE level (Fig. a) High basic total IgE level mast cells reacted less (weaker) upon antigen challenge with decreased histamine release.

Total IgE The total serum IgE level measured either at the optimal day day 11 or at the time of death, shows no significant correlation with the amount of histamine released with antigen from isolated mast cells (Table 2).

Specific IgE antibody The anti-egg albumin IgE antibody level at day 11 shows a significant positive correlation with the histamine release with a correlation coefficient of 0 61 (p=0 003)) (Fig. 2b) However there was no correlation between specific IgE antibody levels at the time of death and the histamine release (Table 2)

Specific IgE antibody/total IgE ratio The correlation of the ratio of specific IgE antibody over total IgE with histamine release is highly significant (r=0.67 p=0.0007) (Fig. 2) and slightly impro ed

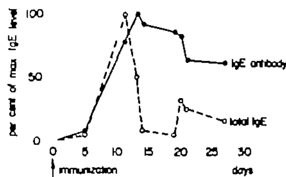


Fig 1 Changes of IgE in rat serum after immunization Total IgE (ng/ml) and anti-egg albumin IgE antibody (units/ml) measured in rat serum at different days after and just prior to immunization with 70 μg of egg albumin in pertussis vaccine

It is a well known fact that rats from some strains are difficult to sensitize to obtain specific IgE antibodies (Jarrett 1978 Karlsson et al 1979*b*) as well as good histamine release from isolated mast cells after challenge with purified antigen without serumfactors (e g phosphatidylserine) (Norn 1968 Sydbom & Uvnäs 1976) We have also seen a rather great variation in the response within a group of animals and it is therefore advantageous to be able to select out the poor or non responders By the use of these techniques (PRIST PRAST) we have tried to find a correlation between IgE levels (total or specific) and anaphylactic histamine release that eventually would enable us to predict the degree of sensitization in one single rat The histamine release studies were performed in an in vitro system using isolated rat mast cells and histamine was measured by a fluorescent technique (Shore et al 1959) The mast cells were isolated from Hooded Lister rats as this strain has been found to give good IgE antibody responses (Bennich et al 1978) which enables the study of anaphylactic histamine release without the addition of exogenous factors like phosphatidylserine (Sydbom et al 1979)

MATERIALS AND METHODS

Egg albumin was purchased from Difco laboratories Detroit Michigan, USA Ficoll from Pharmacia Fine Chemicals AB Uppsala, Sweden, and pertussis vaccine (lot no 120 274) from Statens Seruminstitut Copenhagen Denmark. Inbred Hooded Lister rats originating from rats purchased from Møllegaard A Islaboratorium ApS L. Skensved Denmark were used All other materials were obtained from the usual commercial sources. Due to refined purification methods by many manufacturers, the B pertussis vaccine has lost its adjuvant activity on the IgE production in rats, presumably because of loss of the lymphocytosis promoting factor as reported by Tada et al (1972) However we have found that the Danish manufactured B pertussis still possesses high adjuvant activity

Immunization of rats Male rats were injected s c. with 1 ml of pertussis vaccine together with 70 μg egg albumin per animal Mast cells were usually taken from the rats to 4 weeks after immunization Serum samples were taken from the rats before immunization and at day 5 and 11 after immunization and finally when the rats were killed for isolation of mast cells. About 1 ml of blood was collected by cutting the tail and allowed to clot for 15 min at RT before centrifugation, 15 000–20 000×g for 15 min at 4°C The serum was kept at −70°C before testing.

Determination of IgE in rat serum Total IgE and IgE antibody (egg albumin specific) were determined by solid phase radioimmunoassay techniques earlier described for human IgE (Ceska & Lundkvist 197_, Wide et al 1967 Wide et al. 1977) and modified for rat IgE. Total IgE, as measured by PRIST (paper radioimmunosorbent test) based on paper disc coupled anti rat IgE antibodies of rabbit origin an IgE myeloma standard (IR2) and immunosorbent purified ^{125}I-anti Fc-ε (rat) antibodies is expressed in ng per ml (Karlsson et al 1979*a*). IgE antibody against egg albumin as determined by PRAST (paper radio allergo sorbent test) based on paper disc coupled antigen (egg albumen) egg albumin positive rat standard serum and immunosorbent purified ^{125}I-anti Fc-ε (rat) antibodies was expressed as units per ml where 1 unit of specific IgE antibody corresponded to less than 1 ng of total IgE (Karlsson et al. 1979*b*)

Isolation of mast cells Mast cells were collected from the pleural cavities (Sydbom & Uvnäs 1976) of immunized rats and isolated by density gradient centrifugation on Ficoll according to the method of Thon & Uvnäs (1967). The mast cells were washed three times at 20°C with an isotonic saline solution containing 10% (v/v) Sorensen's phosphate buffer (Na_2HPO_4+KH_2PO_4, 67 mM) pH 6.7 and human serum albumin (1 mg/ml)

Incubation procedure The isolated mast cells were incubated with antigen (10 μg/ml) for 10 min at 37°C in medium and the mast cell concentration was usually about 5 000 cells/ml

The standard incubation medium was an isotonic saline solution (NaCl 137 mM KCl 2 7 mM $CaCl_2$ 1.8 mM, $MgCl_2$ 1 mM) containing 10% (v/v) Sörensen's phosphate buffer (67 mM) pH 6.7 and human serum albumin (0.5 mg/ml) The incubation was terminated by placing the tubes in ice water and centrifuged (350×g) for 10 min at 4°C The supernatants were decanted into new tubes and 0 ml of 0 1 M HCl was added to the cell residues. The supernatants and the cell residues were heated in a boiling water bath for 5 min 1 8 ml of incubation medium was added to the cell residue to readjust the volume to ml. Histamine was determined both in the supernatant and the cell residues by the method of Shore et al (1959) omitting the extraction procedure (Bergendorff & Uvnäs 1972) Histamine release was expressed as percentage of total histamine The spontaneous histamine release has been subtracted from the values presented. The spontaneous histamine release from mast cells of immunized Hooded Lister rats averaged 8 8±0 7% (*n*=__).

Table 2. *Correlation between histamine release and serum IgE*

Total and basic total IgE (ng/ml) and specific IgE antibody (units/ml) determined in Hooded Lister rat serum prior to immunization, at day 11 after immunization or at the death-day is correlated to the histamine release (%) after addition of 8 µg/ml of egg albumin (10 min incubation at 37°C) to isolated mast cells. Partial correlation was calculated as described by Spiegel (1961). The variables are histamine release (1), specific IgE antibody day 11 (2) and basic total IgE (3). The partial correlation coefficient $r_{13.2}$ is then the correlation between histamine release and basic total IgE keeping the specific IgE antibody constant

Correlation between histamine release (1) and	Correlation coefficient	Significance level	Partial correlation coefficient
Basic IgE (3)	0.55		−0.65 ($r_{13.2}$, IgE antibody constant)
Total IgE (d. 11)	−0.38	N.S.	
Total IgE (death-day)	−0.19	N.S.	
IgE antibody (d. 11) (2)	0.61		0.68 ($r_{12.3}$, basic IgE constant)
IgE antibody (death-day)	−0.05	N.S.	
IgE antibody/total IgE (d. 11)	0.67		
IgE antibody/total IgE (death-day)	0.37	N.S.	

N.S. not significant = $0.001<p<0.01$ $p<0.001$

Sydbom et al. 1979). Our original rationale for using Hooded Lister rats was their high basic levels of serum IgE (Karlsson et al. 1979) and the subsequent finding of good responsiveness to antigen challenge with good histamine release from isolated mast cells (Sydbom et al 1979 Pauwels et al 1979).

It was therefore near at hand to assume that high basic total IgE levels would correspond to high histamine releasing capacity However we found that very high basic levels of serum IgE counteracted the histamine release. This could be due to a competition for the mast cell receptors between the basic total IgE present in serum and specific IgE antibody synthetized as a result of the immunization. Basic total IgE may fluctuate in both immunized and nonimmunized animals due to environmental factors (Karlsson *et al* 1979*a*)

As expected the level of specific IgE antibody is significantly correlated to the histamine releasing capacity of the mast cells (Fig. 2*b*). This significant correlation is restricted to the specific IgE antibody on day 11 and cannot be seen for specific IgE antibody on the day of death This might be due to the different half-lives of IgE in serum and on mast cells. Tada et al. (1975) have calculated the half lives to be 12 hours in rat serum and 7.4 days on the mast cell surface This means that the serum levels of specific IgF antibody can be very low even at a time when the mast cells may be loaded and therefore show high histamine releasing capacity The correlation between histamine release and the specific IgE antibody should therefore be more significant when using the maximal specific IgE antibody levels, here on day 11 i.e. the highest specific IgE antibody concentration that the mast cells have been exposed to In view of the competition situation the finding that the ratio of specific IgE antibody over total IgE is somewhat better correlated with the histamine release than specific IgE antibody itself is not unexpected. So in spite of a correlation coefficient not being very impressive it can be concluded that if a rat shows a low basic total IgE level prior to immunization and a high specific IgE antibody level on day *11* after immunization, there is a very high probability for high antigen induced histamine release from isolated mast cells from that rat.

In conclusion we found no correlation between specific IgE antibody on the day of death and histamine release but a significant negative correlation between basic total IgE and histamine release and a significant positive correlation between specific IgE antibody on day 11 and histamine release from isolated sensitized mast cells challenged with antigen. The correlation coefficients are however not very impressive and there could be a number of explanations for this. We have not measured the IgG antibody levels and must take into account the influence they can have on the histamine release situation. Also local cellular differences can be of importance as Yamamoto et al. (1974) have reported that the antigen induced histamine release

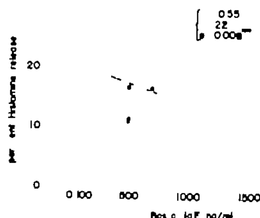


Fig 2a Correlation between basic total IgE and histamine release Basic total IgE (ng/ml) was measured in rat serum collected just prior to immunization with 20 μg of egg albumin in pertussis. Histamine release (%) was measured after addition of 10 μg/ml of egg albumin (10 min incubation at 37°C) to isolated rat mast cells taken between day 1 and 27 after immunization (Basic total IgE was defined as total serum IgE when measured on day 0 or at the latest on day 5 after immunization. There were no significant difference between IgE levels on day 0 and day 5 (Table 1)

than when comparing the specific IgE antibody alone with histamine release This is true for day 11 but there is no significant correlation when using the values from the time of death (Table 2) When three or more variables affect each other and cooperate this can be evaluated either by partial correlation or by forming a ratio between two of the variables Both ways were tested and gave a better

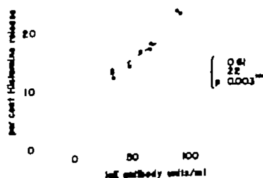


Fig 2b Correlation between specific IgE antibody (day 11) and histamine release. Specific IgE antibody (units/ml) was measured in rat serum collected at day 11 after immunization with 20 μg of egg albumin in pertussis Histamine release (%) was measured after addition of 10 μg/ml of egg albumin (10 min incubation at 37°C) to isolated rat mast cells taken between day 12 and 27 after immunization.

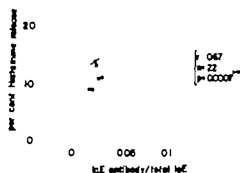


Fig 2c Correlation between specific IgE antibody/tot IgE (day 11) and histamine release The ratio of speci IgE antibody (units/ml) over total IgE (ng/ml) both mea ured in rat serum collected at day 11 after immunizatio with 20 μg/ml of egg albumin (10 min incubation at 37° to isolated rat mast cells taken between day 12 and 27 af Immunization

correlation of about the same magnitude The corr lation coefficient between specific IgE antibod (day 11) and histamine release was 0 61 and i creased to 0 68 when calculated with partial correl tion (Spiegel 1961) and to 0 67 when using a rat (Table 2)

DISCUSSION

The specific IgE antibody in serum from immunize Hooded Lister rats show a maximum between da 11 and 14 (Fig. 1) in agreement with earlier studie (Karlsson 1978) The total IgE time course patter is under the experimental conditions used rathe similar to the specific IgE antibody but declme faster possibly because the increase in total IgE i an effect of adjuvant stimulation of already existin IgE producing cells as after reimmunization an therefore of a different nature than the expressio of IgE antibody after primary immunization

The finding that there is a significant negativ correlation between the basic total IgE before im munization and the histamine release from isolate rat mast cells challenged with antigen was some what unexpected (Fig 2a) Using a pure system fo histamine release studies that is isolated mast cell instead of mixed peritoneal cells a pure antigen an not horse serum and without the addition of phos phatidylserine will result in very low histamine re lease (4–9%) from mast cells originating from Sprague Dawley rats (Stechschulte & Austen 1974

Acta Physiol Scand 1979 107 319–325

Fibre types in human abdominal muscles

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HÄGGMARK T & THORSTENSSON A. Fibre types in human abdominal muscles. Acta Physiol Scand 1979 107 319–325 Received 23 April 1979 ISSN 0001-6772. Department of Physiology III Karolinska Institutet and the Department of Surgery Karolinska Sjukhuset, Stockholm, S eden.

Histochemical muscle fibre composition was studied in biopsies from the four different muscles of the abdominal wall (rectus abdominis, RA, obliquus externus, OE, obliquus internus, OI and transversus abdominis, Tr) in 13 normal human subjects (9 females and 4 males, age 24–55 years) undergoing gall-bladder surgery Muscle fibres were classified as Type I II A, II B or II C on the basis of their myofibrillar ATPases pH lability There were large inter-individual variations in fibre composition, hereas, in general the differences between the different muscles were minor or non xistent. Mean fibre distribution ranges were 55–58% I, 15–23% II A 21–28% II B and 0–1% II C fibres. The least fibre diameters were similar for all types and muscles (range of means 50–54 µm) xcept for T in which the Type II fibres were smaller (mean 45 µm) There was high correlation in the size of Type I vs. II fibres and Type II A vs. II B fibres in all layers. The oxidativ e potential (NADH-diaphorase staining intensity) appeared high in Type I fibres and low in Type II fibres, irrespective of subgroups. Thus, based on histochemical fibre composition, the different abdominal muscles appear to have similar functional capacity However functional differences between individuals were indicated by the large inter-individual variation in muscle fibre distribution.

Key ords Muscle fibre composition, muscle fibre size human abdominal muscles

Human skeletal muscles contain a arying distribution of muscle fibre types most commonly distinguished with the histochemical taining for myofibrillar ATPase and classified as Type I (ST) or Type II (FT), with subgroups II A, II B and II C (see e g. Saltin et al. 1977) The fibre types differ in metabolic profile (Essen et al. 1975 Thorstensson et al. 1977), contractile properties and fatiguability although the direct evidence for these latter aspects is thus far relatively incomplete in humans (see however Eberstein & Goodgold 1968 Buchthal & Schmalbruch 1970, cf also Thorstensson 1976) Thus, the distribution of histochemical fibre types in a muscle gives an indication of its functional capacity cf e g the predominance of Type I fibres in the slow soleus muscle (Gollnick et al. 1974 see also Burke & Edgerton 1975)

The information about fibre distribution in the trunk muscles is scarce Of the four muscles in the abdominal wall the rectus abdominis (RA) has been reported to consist of an approximately even distribution of Type I and Type II fibres but with large individual differences (range 32–65% Johnson et al. 1973 Colling-Saltin 1979) No corresponding data is available on the obliquus externus (OE) obliquus internus (OI) or transversus abdominis (Tr) muscles.

The muscles of the abdominal wall control the movements of the trunk and regulate the pressure within the abdominal cavity One important function of this pressure, which may exceed the intrathoracal pressure may be to transform the abdominal cavity into a shock-absorber that helps the spinal column to support part of the static and dynamic load of the upper part of the trunk (e g. Bartelink 1957 Morris et al 1961 Grillner et al 1978) In both trunk control and pressure regulation there appear to be differences between the individual abdominal muscles RA, OE and OI perform ventroflexion whereas OE and OI are mainly responsible for lateral flexion and rotation (Floyd & Sil er 1950 Partridge & Walters 1959). The differences in

was different in different target organs tested The response of a single mast cell is therefore probably the result of the histamine releasing capacity of the single cell as well as the IgE situation on the mast cell surface local differences and dependence of other factors and other cells

This study was supported by grants from the Swedish Medical Research Council (B77-04X 39-13A B78-04X 39-14B and project No 13X 3556)

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Table 1 *Mean alues (±1 S.D) and ranges for the different muscle fibre characteristics studied in m recti abdominis (RA), m obliquus externus (OE), m. obliquus internus (OI) and m trans ersus abdominis (Tr)*

	RA	OE	OI	Tr
% Type I fibres	55±8 39–68	58±11 40–77	56±13 36–74	56±4 51–64
% Type II A fibres	22±9 9–36	21±13 7–45	23±11 13–45	15±8 6–29
% Type II B fibres	24±5 17–33	21±8 9–32	21±9 5–37	28±8 1. 37
Diam. Type I fibres, µm	50±10 35–67	50±8 36–63	50±14 32 71	49±10 31–61
Diam. Type II A fibres, µm	52±14 34–83	52±14 37 71	51±15 34–82	47±9 31–62
Diam. Type II B fibres, µm	52±15 34–84	54±12 33–73	52±15 31–75	43 _8 29–55
... ratio ... I/Type II fibres	0.99±0.34 0.45–1 60	0.88±0.22 0 55–1.28	0 96±0.26 0 64–1 38	1.23±0.25 0 97–1 71
...d area ... Type I fibres, %	54±14 29–73	54±14 35–72	55±18 28–85	60±7 52–75

...ectively evaluate the occurrence of fibre grouping (cf. Johnson et al. 1973) No difference was observed between males and females.

The distribution of the two main types of muscle fibres, Type I and II, was similar in all four muscle layers (mean range 55–58% Type I fibres Table 1) However within each muscle layer the variation in fibre composition was large between individuals (Fig. 2) especially for the RA, OE and OI muscles (total range 36–77% Type I fibres, Table 1). There was a significant correlation between % Type I fibres in the RA, OE and OI muscles (=0 6–0 8 P<0 01). Three persons showed a predominance of Type II fibres in these muscles (Fig. 2) In the Tr muscle, on the other hand a smaller range of fibre distribution was observed and none of the eight cases showed a predominance of Type II fibres (Fig. 2). The distribution of Type I fibres in Tr was not correlated to that of the other abdominal muscles.

Also, with respect to the distribution of subgroups of the Type II fibres (II A and B), no significant differences were seen between the different muscle layers. Large variations between individuals were observed in each layer (Table 1) There was no difference between % Type II A and II B fibres for RA, OE and OI muscles. In Tr however the % II B fibres was higher than the % II A fibres in 7 of the 8 subjects (the difference was significant at the 5% level) and a high % II B fibres also corresponded to a low % II A fibres in the Tr muscle (r=–0.86, P<0 01) Relating percentage distribution of Type II subgroups to % Type I fibres significant positive correlation coefficients (r=0.70–0.87 P<0 01) were found between % II A and % I fibres within the RA OE and OI muscles.

There were no differences between the different abdominal muscles with respect to Type I fibre size

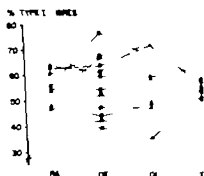


Fig 2 Individual values for the relative distribution of Type I fibres in m. recti abdominis (RA), m. obliquus externus (OE), m. obliquus internus (OI) and m transversus abdominis (Tr). The values for each individual are connected with thin lines.

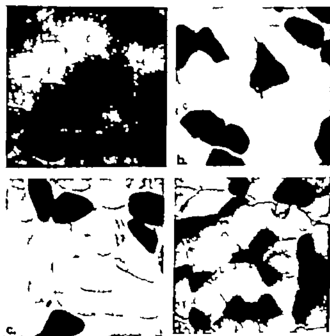


Fig 1 Cross-sections of muscle biopsy specimens stained for myofibrillar ATPase after preincubation at pH 10.3 The biopsies were taken from (*a*) m rectus abdominis, (*b*) m obliquus externus, (*c*) m. obliquus internus, and (*d*) m transversus abdominis in the same person. (Type I fibres stain light and Type II fibres dark with this procedure.)

anatomical arrangement between the muscles also influence their involvement in the production of intraabdominal pressure (IAP) where the electromyographic activity of RA for example appears to be less closely related to changes in IAP (Bartelink 1957 Morris et al 1961 Grillner et al 1978) In addition it has been shown that the abdominal muscles are active to maintain postural stability (e g. Klausen 1965) and during a variety of natural movements including locomotion (Grillner et al 1978 and unpubl)

The present study was undertaken to investigate the intra- and interindividual differences in muscle fibre composition in the four muscles of the abdominal wall to obtain a basis for evaluation of their specific functional capacities.

MATERIAL AND METHODS

Muscle specimens were obtained during surgery from 13 subjects 9 females and 4 males. Their age and weight ranged between 4-55 years (median 44 years) and 57 76 kg (median 60 kg) respectively The patients were well informed before the operation and gave their consent to participate.

The subjects were of normal body constitution and did not suffer from any neuromuscular disorder They were all undergoing gall-bladder surgery All were operated with an oblique incision 10-15 cm long, beneath the costal margin on the right side The different muscle layers were identified as they were cut and the biopsies (approx. 0 5 g were taken surgically from the different muscles, m. rectus abdominis (RA) m. obliquus externus abdominis (OE), m. obliquus internus abdominis (OI) and m. transversus abdominis (Tr) Due to technical difficulties, biopsies from Tr were obtained in only 8 cases (5 females and males).

The biopsies were directly prepared for histochemical analysis and frozen in isopentane cooled with liquid nitrogen. Until further analysis was performed, the muscle samples were kept at −80°C in a freezer

The histochemical analyses were carried out on 10 µm thick serial cross-sections cut in a cryostat at −2°C Muscle fibre types were classified on the basis of the pH lability of their myofibrillar ATPases (Brooke & Kaiser 1970*a* and *b*) Serial sections were preincubated at pH 10.3 and 4.3 to differentiate the two main fibre types Type I (acid stabile ATPase) and Type II (alkaline stabile ATPase) and at pH 4 6 to differentiate the subgroups of Type II fibres. Type II A and Type II B fibres, Type II A having a more acid labile myofibrillar ATPase and thus staining lighter than the Type II B fibres at pH 4 6 (cf. Fig 4) Undifferentiated fibres, i e fibres showing an even dark staining intensity at all pH-levels, were called Type II C according to Brooke & Kaiser (1970 a). No muscle sample contained less than 700 fibres. The average number of fibres counted from each biopsy was 785 (± 47). The oxidative potential of the fibres was estimated on serial sections with the histochemical staining method for NADH-diaphorase (Novikoff et al. 1961). The staining intensity of the fibres was arbitrarily and subjectively classified as dark medium or light and the sections were then matched with the ATPase stain (pH 4 6) for fibre type identification Comparison of staining intensity was done on each muscle sample separately no evaluation of absolute staining intensity was performed between layers or subjects.

Fibre sizes were estimated with the method of least diameter (Dubowitz & Brooke 1973). A minimum of 30 fibres was included in the calculation of mean least diameter for each fibre type Assuming circular shaped fibres the area ratio between Type I and Type II fibres was calculated as well as the relative area occupied by Type I fibres (see Gollnick et al 1972 and Thorstensson 1976).

Differences were tested for significance with Student's *t*-test

RESULTS

All four of the abdominal muscles demonstrated a mosaic pattern of Type I II A and II B fibres (II C fibres comprised only 0-3 % of the total number of fibres) The fibre types appeared evenly distributed throughout the muscle sample cross sections (cf Figs. 1 and 4) No attempt was however made to



Fig. 4. Serial sections of muscle biopsy from the obliquus internus muscle *stained for (a) myofibrillar ATPase after* preincubation at pH 4.6 (Type I and IIB fibres stain dark and Type II A fibres stain light) and (*b*) NADH diaphorase. Some corresponding fibres are identified and marked in the two pictures.

ing-Saltin (1979) reported that RA contained about twice as many Type II A fibres as Type II B fibres, which is similar to what has earlier been found for the vastus lateralis muscle (see Saltin et al 1977). In the present study nearly equal proportions of II A and II B fibres were seen in RA as well as OE and OI whereas Tr demonstrated an even higher percentage Type II B fibres. Tr also differed from the other layers with respect to fibre size (least diameter), showing smaller Type II fibres. In general, however the fibre sizes in the abdominal muscles (range 43–54 μm) were in good agreement with those earlier *reported for other normal human* skeletal muscles (for refs see Saltin et al 1977) including RA, for which muscle Polgar et al (1973) found average least diameters of 43 μm (Type I fibres) and 55 μm (Type II fibres) in 6 subjects investigated post mortem.

Thus, the abdominal muscles appear to have similar characteristics to the majority of the other human skeletal muscles with respect to fibre type distribution. The deviations seen in Type II subgroup composition and fibre size particularly in Tr might be related to the activity pattern of the muscles (and of the subjects in general). All the subjects in the study had a relatively low level of daily physical activity although none of them was ex*tremely inactive. Increases in the relative number* of Type II A fibres and in the size of a specific fibre type have been reported in connexion with physical

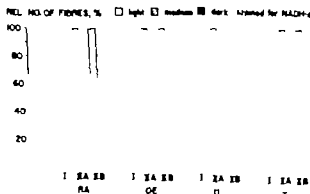


Fig. 3. Mean relative number of muscle fibres of each type and within each individual muscle that were independently rated as having dark, medium or light staining intensity for NADH-diaphorase. Abbreviations are the same as in Fig. 2.

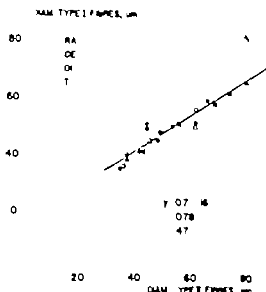


Fig 3 The relationship between the mean values for least fibre diameter of Type I and Type II fibres in the four abdominal muscles in the different subjects. Abbreviation and symbols are the same as in Fig. Line of identity is given along with the linear regression line for all points (=0 78 $P<0\ 01$)

(least diameter Table 1) whereas the Type II fibres (both II A and B) were smaller in Tr than in the other layers ($P<0\ 05$ cf Fig. 1) Mean values and ranges are shown in Table 1 A comparison of the least diameters of the different fibre types within the different layers showed significant ($P<0\ 05$) differences only for OE in which Type II fibres were larger than Type I fibres and for Tr which showed the opposite pattern and in which muscle also the II A fibres were larger than the II B fibres Within all layers there were highly significant correlations in fibre sizes between Type I and II fibres ($r=0\ 86$–$0\ 97$ $P<0\ 01$ Fig 3) as well as between Type II A and II B fibres ($r=0\ 76$–$0\ 87$ $P<0\ 01$)

The calculated ratio between Type I and II fibre area demonstrated large differences between individuals (Table 1) However there were no significant differences between the RA OE and OI muscles The Tr muscle had a higher Type I/Type II area ratio than the other muscles (see above) Consequently the relative distribution of Type I fibres (% Type I area Table 1) was higher for the Tr muscle than shown by just the percentage value However there were still no significant differences (or correlations) in fibre distribution (% Type I area) between Tr and the other abdominal muscle layers.

The majority of the Type I fibres (86-100 stained dark for NADH-diaphorase compared the other fibre types in all layers (Figs. 4 and 5). N Type I fibres were found with a light staining inte sity Relatively small differences were present b tween II A and II B fibres, the majority of bo subgroups stained light for NADH-diaphorase in a layers (Figs 4 and 5) Not more than a third of th II A fibres had a medium staining intensity in any the samples tested Type II B fibres with a mediu staining intensity were found only occasionall (Fig 5)

DISCUSSION

All four muscle layers of the anterior abdomina wall contained a mosaic of Type I and Type I fibres without obvious signs of fibre grouping. The proportion of Type I and Type II fibres was abou equal in all muscle layers, the mean range was 55-58% Type I fibres. No differences were observed between males and females but large interindividual differences in fibre composition were present These observations on the abdominal muscles are similar to what has earlier been reported for the majority of skeletal muscles studied in normal man (e g Johnson et al 1973) Some exceptions exist, however e g the soleus muscle which shows a consistent predominance of Type I fibres (Gollnick et al 1974 see also Saltin et al 1977)

The fibre compositions of RA OE and OI were shown to be correlated with one another Individuals could be found with either a predominance of Type I (slow) or Type II (fast) fibres in these muscles It is not known if for example slow abdominal muscles correspond also to slow characteristics of other muscles in the same individual. Tendencies towards such intraindividual correlations in fibre composition between different muscles have been reported for some extremity muscles (see Saltin et al 1977) However these studies were limited to a small number of subjects investigated post mortem

Up to now fibre composition of the abdominal muscles has only been studied in the RA muscle The present finding of a mean of 55 Type I fibres in RA is in good agreement with the values earlier obtained by Johnson et al 1973 (46) and Colling Saltin 1979 (51%) Both authors also report large differences between individuals (3 -76%) Col-

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training (Henriksson 1976 Thorstensson 1976) A relatively low level of utilization of the abdominal muscles particularly the Type II fibres might also be the explanation for the low oxidative potential seen in the Type II A fibres as compared to what is normally found in extremity muscles (cf Essén et al 1975 Henriksson 1976)

The abdominal muscles function to control the trunk both during posture (e g Klausen 1965) and natural movements including locomotion (Grillner et al 1978 and unpubl) This includes the regulation of the intraabdominal pressure (IAP) This pressure can act to support the upper part of the body and thereby take up part of the load on the spine (for discussion and refs see Grillner et al 1978) The differentiation of the abdominal wall into different individual muscles with varying main fibre directions makes possible a refined control of the movements of the trunk The coordination of the different muscles varies in different movements as evidenced by the electromyographic activity (EMG) (Floyd & Silver 1950 Partridge & Walters 1959 Carlson et al unpubl) The differences in anatomical arrangement seem to have an effect also on the involvement of the individual muscle layers in regulating the IAP From an anatomical point of view the Tr appears to have a mechanical advantage in the production of IAP since it is forming a girdle around the abdominal cavity EMG recordings with surface electrodes placed over the OE muscles (probably also picking up signals from the OI and Tr) have shown close relations with changes in IAP (Bartelink 1957 Morris et al 1961 Grillner et al 1978) whereas the RA activity appeared less consistent in this respect Thus functional differentiation exists between the different abdominal muscles However this does not appear to correspond to a differentiation between the layers in terms of fibre distribution The even distribution of fibre types indicates a functional capacity for both fast contractions and endurance in each muscle layer This arrangement appears appropriate in light of the wide spectrum of demands that are put on the abdominal muscles during natural movements These functional demands may include maximal isometric contraction as in lifting (e.g. Bartelink 1957 Morris et al 1961) fast compensatory contractions as in walking down from steps or repeated submaximal contractions in association with the IAP changes occurring with each step during normal locomotion (Grillner et al 1978)

The large differences in fibre type distribution between individuals indicate differences in performance capacities It is conceivable that all else being equal a person with a high proportion of Type II (particularly Type II B) fibres in the abdominal muscles will be more affected by fatigue and thus be less capable of producing any appropriate fast corrective movements of the trunk or accomplish an adequate intraabdominal pressure On the other hand a low proportion of Type II fibres in the abdominal muscles could also under non-fatigued conditions decrease the capacity to produce a fast rise in tension (and IAP) in response to sudden loads on the body e.g walking down the stairs or jumping down from a height In both situations this inadequacy in the functional capacity of the abdominal muscles due to an extreme muscle fibre composition would lead to an increased mechanical load on the spine which may in turn be a detrimental factor in the etiology of back pain (cf Nachemson 1971)

The authors are indebted to Å Arvidson, M Lindqvist and M Wilén for skillful technical assistance and to Drs H Carlson S Grillner and M Zomlefer for valuable comments on the manuscript.

The study was supported by grants from the Swedish Defense Research Organization (Project No. 11657) and the Research Council of the Swedish Sport Federation.

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The effect of propranolol on the serotonin concentration in the portal plasma after vagal nerve stimulation in the cat

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PETTERSSON G. AHLMAN H BHARGAVA, H. N DAHLSTRÖM, A. KEWENTER, J LARSSON I & SIEPLER, J K. The effect of propranolol on the serotonin concentration in the portal plasma after vagal nerve stimulation in the cat. Acta Physiol Scand 1979 107 327–331 Received 26 April 1979 ISSN 0001-6772. Department of Surgery III and Institute of Neurobiology University of Göteborg, and Department of Pharmacology and Pharmacognosy College of Pharmacy University of Illinois, Chicago, USA.

Efferent cervical vagal nerve stimulation in the cat caused marked increase of the portal plasma 5-HT concentration This increase was more than two-fold within 15 min of stimulation After cessation of stimulation portal plasma 5-HT returned to basal levels within 10 min. Treatment with the β-adrenoceptor antagonist propranolol, in various doses (0 1–. mg/kg b.w.t.), did not abolish but significantly reduced the response to vagal stimulation, particularly during the final part of the stimulation period The results confirm the existence of β-adrenoceptor-mediated release of 5-HT but also suggest that other mechanisms for 5-HT release may be involved in the response on vagal nerve stimulation.

Key words Portal plasma, serotonin fluorimetric assay propranolol, vagal nerve stimulation, cat

The serotonin (5-HT) in the gut is mainly stored in the enterochromaffin cells (EC) of the mucosa (Benditt & Wong 1957 Penttila 1966) The EC is a basal-granulated entero-endocrine cell with amine handling properties according to the APUD concept (Pearse 1969) Different populations of EC have been demonstrated to contain a polypeptide, e.g. motilin, substance P or enkephalin in addition to the amine 5-HT (Pearse et al 1974 Pearse & Polak 1975 Alumets et al 1978).

EC may release their content of 5-HT after luminal stimuli, such as mechanical stimulation, low pH or hypertonic glucose. However evidences for a neural release mechanism have also been presented (Burks & Long 1966, Hohenleitner et al 1971 Tansy et al. 1971 Ahlman et al. 1976). This release appears to be mainly adrenergic in nature and it is likely that β-adrenoceptor mechanism is involved (Ahlman et al. 1976*b* Pettersson et al. 1978, 1979). These previous studies were made using a cytofluorimetric method where the 5-HT content in individual EC was studied after in vivo vagal nerve stimulation (Ahlman et al 1976*a*, *b*), after in vitro incubations with adrenergic drugs (Pettersson et al. 1978) or transmural field stimulation (Pettersson et al 1979).

The present investigation was performed to further study the importance of the postulated β-adrenoceptor mechanism for the release of 5-HT from EC into the portal circulation upon vagal nerve stimulation in vivo using various doses of the β-adrenoceptor antagonist, propranolol

MATERIAL AND METHODS

Animals and blood sampling 23 adult cats of both sexes were used. Prior to the experiments the animals were

Table 1. The 5-HT concentrations (ng/ml) in portal plasma of untreated cats, cats with ligated adrenals and cats treated with propranolol (various doses) before, during and after efferent vagal nerve stimulation. Mean values and S.E.M. Difference from control calculated from the changes in 5-HT levels indicated by * ($P<0.05$)

	Initial levels	During vagal n. stimulation			After vagal n. stimulation		
	0'	5'	10'	15'	20'	25'	30'
Control (n = 7)	197±17	326±38	443±49	487±56	309±19	208±29	181±10
Adrenals ligated (n = 4)	203±19	275±19*	361±22*	483± 8	325±20	272±15	223±10
Propranolol, 2 mg/kg (n = 6)	267±24	364±19	501±35	257±15	237±19*	318±28	328±31
Propranolol, 1.05 mg/kg (n = 3)	191±38	256±61	318±14	220±31	232±24	265±20	259±35
Propranolol, 0.105 mg/kg (n = 3)	240±48	332±37	402±41	285±48	281±48	265±52	341±34

In cats with ligation of the suprarenals the 5-HT response was slower in onset but reached the same maximum level at 15 min stimulation as in the control group (Table 1).

Propranolol did not completely block but reduced and altered the response to vagal stimulation at all three doses tested (Fig. 1 and Table 1). The peak 5-HT levels was now seen after 10 min stimulation (instead of at 15 min as in the control group) followed by a return of plasma 5-HT to basal levels at 15 min stimulation. The difference from control (stimulation without propranolol treatment) was statistically significant ($P<0.05$) for propranolol 2.0 mg/kg already at 5 min stimulation and for all three propranolol groups at 15 min (end of stimulation) (Table 1). During the first 10 min of stimulation the blockade after the highest dose of propranolol seemed to be less effective although no significant difference between the various doses of propranolol could be demonstrated. The reduction from control of the 5-HT levels after propranolol (groups taken together), calculated by difference in curve areas (Fig. 1), was about 50%. In the propranolol groups there was a minor but constant secondary increase of the portal plasma 5-HT after the end of stimulation at 25 and 30 min.

DISCUSSION

The present study demonstrates a rise of the 5-HT concentration in portal plasma after vagal nerve stimulation. This effect cannot be explained by changes in blood flow, since splanchnic blood flow is not reduced but rather increased after vagal stimulation (Kewenter 1965, Martinsson 1965). The increase in 5-HT concentration on vagal stimulation is antagonized by propranolol, most obviously during the final part of the stimulation period. Since even the lowest dose of propranolol tested (0.1 mg/kg) was effective in this respect, a specific β-adrenoceptor mechanism seems probable.

As shown in Fig. 1 the 5-HT concentrations in portal plasma during the first 10 min of vagal nerve stimulation in propranolol pretreated cats were still very high. This may be due to a propranolol resistant release of 5-HT, to an initially reduced blood flow, or a combination of the two alternatives. A reduction of blood flow is likely to occur after propranolol. Furthermore, through its membrane stabilizing effect propranolol inhibits the uptake of 5-HT into platelets. Oral administration of propranolol (3 mg/kg) reduced the 5-HT uptake into platelets by 50% in human expts. (Grobecker et al. 1973). Similar concentrations of propranolol are probably reached after 2 mg i.v. in the present expts. Under normal conditions more than 50% of the 5-HT released from the gut is taken up into platelets before reaching the liver (Jaffe et al. 1977) indicating that inhibition of this mechanism by propranolol may contribute to an increase of the measured portal plasma 5-HT levels. Thus, even if the high dose of propranolol further decreased the 5-HT release from EC, this effect may have been masked by the inhibition of the platelet 5-HT uptake resulting in high plasma 5-HT levels.

The present results are in accordance with the previous cytofluorometric findings that the decrease in intracellular levels of 5-HT in EC after efferent stimulation of the vagal nerves was blocked by propranolol pretreatment. Removal of the superior cervical sympathetic ganglia one week prior to the experiments also blocked the vagally induced 5-HT decrease in EC, indicating that adrenergic nerve fibres from these neck ganglia were mediating this

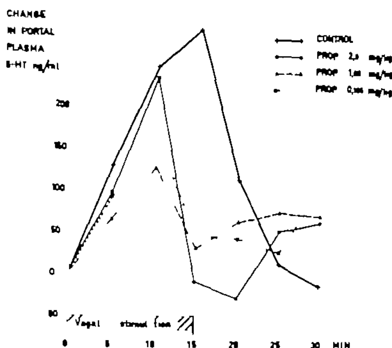


Fig 1 Diagram illustrating the changes in portal plasma 5-HT concentrations (mean values) on efferent vagal nerve stimulation in untreated cats and cats treated with various doses of propranolol based on the values given in Table 1

fasted for 24 h but had free access to water Anesthesia was induced with ether and maintained with chloralose (50 mg/kg of b wt) Blood samples (? ml each) for assays of 5-HT were drawn before during and after nerve stimulation (according to the time schedule in Fig. 1) through a heparinized catheter inserted into the portal vein with the tip in the liver hilus region For volume replacement all animals were given 10 ml of physiological saline through the femoral vein before the initiation of electrical stimulation and additional 10 ml was given during stimulation Blood samples were stored on ice during the expts and then centrifuged The plasma wa kept frozen until the assays

N r e tim l tion The vagal nerves were dissected free at the cervical level and cut The distal ends were placed in circular electrodes insulated on the outside A square wave impulse generator (Grass Instruments Co Model S4) was used for the electrical stimulation Both vagal nerves were stimulated simultaneously for 15 min with supramaximal parameters. 20 V 2 ms, 8 Hz. The efficiency of the nerve stimulation was judged by the presence of bradycardia fall in blood pressure and small bowel contractions.

Experimental groups 7 cats served as controls In 4 cats the adrenals were bilaterally ligated 12 cats were given propranolol (d l propranolol Inderal® ICI) i in 3 different doses. 2 mg/kg b wt as a bolus dose 20 min before the stimulation (6 cats) 0 75 mg/kg b wt. as a bolus dose ?0 min before the stimulation and in addition 0 3 mg/kg b wt /15 min as a constant infusion during the stimulation (3 cats) 0.075 mg/kg b wt. as a bolus dose 20 min before the stimulation and in addition 0 03 mg/kg b wt./15 min as a *constant* infusion during the stimulation (3 cats)

Assay of 5-HT in plasma The plasma concentration of 5-HT was determined by the modified fluorimetric method of Cox & Perhach (1973) for brain tissue Briefly the method consisted of vortexing 250 µl of plasma with 5 ml n-butanol for 1 min followed by shaking for 5 min on a mechanical shaker at 160 oscillations per minute. The mixture was then centrifuged at 3 000 rpm for 5 min at 4°C. A 2.2 ml aliquot of butanol phase was transferred to a tube containing 5 ml of n-heptane and 1.2 ml of 0.1 N HCl containing 0 1% cysteine. The mixture was shaken for 5 min at 160 oscillations per min and then centrifuged at 3 000 rpm for 5 min One ml of aqueous phase was withdrawn and mixed with 0 4 ml of 0.012% o-phthaldialdehyde in 10 N HCl The mixture was heated in boiling water for 10 min After cooling, the fluorescence of the sample was read at 470 nm after being excited at 360 nm on an Aminco-Bowman pectrofluorimeter The recovery of 5-HT ranged from 72–81% and the assays were reproducible w thin 5%

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RESULTS

In the *control* cats ($n = 7$) the vagal nerve stimulation caused a prompt and steady increase in the portal plasma 5-HT levels up to a maximum of ∠50% of initial levels after 15 min stimulation (Table 1 and Fig 1) The increase was significant already after 5 min ($P < 0.05$) After cessation of stimulation the 5-HT values were normal within 10 min In the 4

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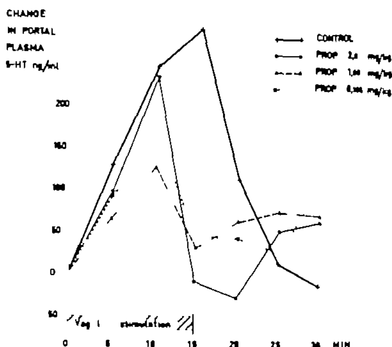


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In the *control* cats ($n = 7$) the vagal nerve stimulation caused a prompt and steady increase in the portal plasma 5-HT levels up to a maximum of 250% of initial levels after 15 min stimulation (Table 1 and Fig 1) The increase was significant already after 5 min ($P<0.05$) After cessation of stimulation the 5-HT values were normal within 10 min In the 4

Table 1. The 5-HT concentrations (ng/ml) in portal plasma of untreated cats, cats with ligated adrenals and cats treated with propranolol (various doses) before, during and after efferent vagal nerve stimulation

Mean values and S.E.M. Difference from control calculated from the changes in 5-HT levels indicated by * ($P<0.05$)

	Portal levels 0′	During vagal n. stimulation 5′	10′	15	After vagal stimulation 20′	25′	30′
Control (n 7)	197±17	326±38	443±49	487±56	309±19	298±?9	181±10
Adrenals ligated (n 4)	202±19	273±19*	361±22*	483±8	325±20	272±15	223±10
Propranolol, 2 mg/kg (n 6)	267±4	364±19	501±35	257±13	237±19*	318±29	328±31
Propranolol, 1 05 mg/kg (n 3)	191±38	256±61	318±14	220±31	25±4	265±20	259±33
Propranolol, 0 105 mg/kg (n=3)	40±48	332±37	402±41	285±48*	281±48	265±52	341±34

cats with ligation of the suprarenals the 5-HT response was slower in onset but reached the same maximum level at 15 min stimulation as in the control group (Table 1)

Propranolol did not completely block, but reduced and altered the response to vagal stimulation at all three doses tested (Fig. 1 and Table 1). The peak 5-HT levels was now seen after 10 min stimulation (instead of at 15 min as in the control group) followed by a return of plasma 5-HT to basal levels at 15 min stimulation. The difference from control stimulation without propranolol treatment) was statistically significant ($P<0.05$) for propranolol 1.05 mg/kg already at 5 min stimulation and for all three propranolol groups at 15 min (end of stimulation) (Table 1). During the first 10 min of stimulation the blockade after the highest dose of propranolol seemed to be less effective although no significant difference between the various doses of propranolol could be demonstrated. The reduction from control of the 5-HT levels after propranolol (groups taken together) calculated by difference in curve areas (Fig. 1) was about 50%. In the propranolol groups there was a minor but constant secondary increase of the portal plasma 5-HT after the end of stimulation at 25 and 30 min

DISCUSSION

The present study demonstrates a rise of the 5-HT concentration in portal plasma after vagal nerve stimulation. This effect cannot be explained by changes in blood flow since splanchnic blood flow is not reduced but rather increased after vagal stimulation (Kewenter 1965, Martinsson 1965). The increase in 5-HT concentration on vagal stimulation is antagonized by propranolol most obviously during the final part of the stimulation period. Since even the lowest dose of propranolol tested (0.1 mg/kg) was effective in this respect, a specific β-adrenoceptor mechanism seems probable.

As shown in Fig. 1 the 5-HT concentrations in portal plasma during the first 10 min of vagal nerve stimulation in propranolol pretreated cats were still very high. This may be due to a propranolol resistant release of 5-HT to an initially reduced blood flow or a combination of the two alternatives. A reduction of blood flow is likely to occur after propranolol. Furthermore, through its membrane stabilizing effect propranolol inhibits the uptake of 5-HT into platelets. Oral administration of propranolol (3 mg/kg) reduced the 5-HT uptake into platelets by 50% in human expts. (Grobecker et al. 1973). Similar concentrations of propranolol are probably reached after 2 mg i.v. In the present expts. Under normal conditions more than 50% of the 5-HT released from the gut is taken up into platelets before reaching the liver (Jaffe et al. 1977) indicating that inhibition of this mechanism by propranolol may contribute to an increase of the measured portal plasma 5-HT levels. Thus, even if the high dose of propranolol further decreased the 5-HT release from EC, this effect may have been masked by the inhibition of the platelet 5-HT uptake resulting in high plasma 5-HT levels.

The present results are in accordance with the previous cytofluorimetric findings that the decrease in intracellular levels of 5-HT in EC after efferent stimulation of the vagal nerves was blocked by propranolol pretreatment. Removal of the superior cervical sympathetic ganglia one week prior to the experiments also blocked the vagally induced 5-HT decrease in EC, indicating that adrenergic nerve fibres from these neck ganglia were mediating this

response (Ahlman et al 1976*b*) The previous cytofluorimetric and the present studies together indicate that vagal nerve stimulation causes a release of 5-HT from EC into the portal blood and that this effect is at least partly mediated via a β-adrenoceptor mechanism Other neural mechanisms may also release 5-HT from EC as studied in the rat (Tobe et al 1976) In vitro studies of the rat duodenum by cytofluorimetry after drug incubations not only supported the probable existence of a β-adrenergic release mechanism for 5-HT from EC but also suggested a cholinergic mechanism (Pettersson et al 1978) However in the cat atropine could not block the decrease of 5-HT in EC after vagal stimulation (Ahlman et al 1976*b*) indicating that in this species the cholinergic mechanism is of less importance

Using fluorescence microscopy catecholamine containing nerve fibres have been demonstrated in the cervical vagal nerve (Muryobayashi et al 1968) Adrenergic nerve terminals have also been observed near EC in the intestinal crypts (Ahlman et al 1976*a*) Part of these terminals probably emanate from the cervical sympathetic ganglia via the vagal nerve as indicated by studies using the retrograde HRP tracing technique (Lundberg et al 1978) Ultrastructural studies have demonstrated autonomic nerve endings of cholinergic adrenergic and peptidergic types near the base of most EC in the crypts of the guinea pig duodenum (Lundberg et al 1978) Furthermore a true synapse between a probable adrenergic terminal and a basal-granulated gut endocrine cell was recently demonstrated in the rat (Newson et al 1979) Thus a morphological basis for an adrenergic neural control of the EC via the vagal nerves has been established In addition circulating catecholamines released from the adrenals at the fall in blood pressure may also contribute to release of 5-HT from EC at vagal nerve stimulation. However in the four cats with bilateral ligation of the adrenals the 5-HT level after 15 min of stimulation was the same as in controls although the initial rise was slower (Table I) This clearly indicates the importance of the direct neural influence on the 5-HT release

A propranolol resistant release of 5-HT on vagal nerve stimulation may have several explanations (*a*) It may be mediated via nonadrenergic neural mechanisms (see above) (*b*) A 5-HT release from serotoninergic nerves in the gut is less probable since Ahlman & Enerbäck (1974) could find no objective evidence for the presence of 5-HT in the myenteric plexus of the guinea pig. (*c*) The possibility of an indirect stimulation of the EC by increased intraluminal pressure or acid pH in the duodenum (Bulbring & Crema 1959 Resnick & Gray 1962) after vagal nerve stimulation cannot be ruled out

The results of the present study confirm the existence of a β-adrenoceptor mediated release of 5-HT However the results also suggest that other mechanisms for 5-HT release may be involved in the total response on vagal nerve stimulation.

Supported by the Swedish Medical Research Council (Grants nos 17X-05220 04X-207 04P-4173); the Goteborg Medical Society the Swedish Society of Medical Sciences the Medical Faculty University of Göteborg the M Bergvall's Foundation and H and G Jeansson s Foundation The technical assistance of Mrs H Andersson is gratefully acknowledged.

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Acta Physiol Scand 1979 107 333–337

Adenosine 3' 5' cyclic monophosphate calcium and magnesium excretion in ethanol intoxication and hangover

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LINKOLA, J FYHRQUIST F YLIKAHRI, R. Adenosine 3' 5' cyclic monophosphate, calcium and magnesium excretion in ethanol intoxication and hangover Acta Physiol Scand 1979 107 333-337 Received 27 April 1979 ISSN 0001-6772. Minerva Institute for Medical Research, Helsinki, and Third Department of Medicine, University of Helsinki, Finland.

Effect of ethanol on adenosine 3' 5' cyclic monophosphate (cAMP), calcium (Ca) and magnesium (Mg) excretion was studied in controlled clinical conditions in man. Seven male volunteers served as their own controls. In 5 subjects cAMP excretion was primarily suppressed by ethanol. Ethanol appeared to have a biphasic effect on Ca excretion, an initial stimulation followed by a conservation phase. Mg excretion was stimulated by ethanol in 5 subjects Subjects having nausea and vomitus and the most severe hangover symptoms had the lowest urinary Ca excretion and the lowest initial cAMP excretion. Ca and Mg metabolism and the susceptibility of the body to the toxic effects of ethanol appeared to be interrelated.

Key words. Cyclic AMP calcium, magnesium, ethanol

Adenosine 3' 5' cyclic monophosphate (cAMP) acts as a renal cellular messenger for parathormone (PTH), which regulates phosphate and calcium (Ca) metabolism (Chase & Aurbach 1967 Massry & Coburn 1973) In addition renal tubular transport of Ca appears to share a common reabsorptive mechanism with magnesium (Mg) (Samly et al 1968, Coburn et al 1970)

Ethanol may acutely increase serum Ca and inorganic phosphate concentration, but also urinary excretion of these ions in man (Kalbfleisch et al. 1963, Markkanen & Nanto 1966) On the other hand, chronic human alcoholics frequently display abnormal serum Ca values (Martin et al 1959 Fankushen et al 1964). Furthermore ethanol has been shown to induce hypomagnesemia (Flink et al. 1954 Heaton et al 1962), which reportedly is a consequence of urinary Mg loss (Heaton et al. 1962, Kalbfleisch et al. 1963). Because recent observations (Linkola et al. 1978 Linkola et al. 1979) indicated biphasic effects of ethanol on water and electrolyte balance we measured stepwise urinary excretion of cAMP Ca and Mg during ethanol intoxication and hangover in controlled clinical conditions.

MATERIALS AND METHODS

The experiments were carried out in a metabolic ward as described in previous paper (Linkola et al. 1978, Linkola et al. 1979). Seven healthy men, age 23–35 volunteered for the experiments. Isocaloric diet (about 30 kcal day^{-1} kg^{-1}) containing 100 mmol sodium (Na) and 90 mmol potassium (K) per day was eaten for 5 days. In the morning of the 5th day the subjects received 2/3 the calories of that day and all Na as NaCl. Then, beginning at noon, they fasted to 6 p m. and were supine for 22 h. From 6 p.m. to 9 p.m. they received deionized water 10 ml/kg. At 8 a.m. next morning they received water 5 ml/kg. At 10 a.m. they assumed standing position walking slowly around. They had nothing to eat until noon. After that they had a meal (about 30 kcal/kg, 100 mmol Na and 90 mmol K)

Beginning in the morning of the 7th day the same procedure as two days earlier was repeated, except that water in

Table 1 *Urinary cAMP excretion in subjects I–VII in µmol/g creatinine during contr l (C) and ethanol (E) experiment*

	C				E					
	p.m		a.m		p m		a.m			
Subject	6–9	9–12	0–6	6–12	6–9	9–1	0–6	6–1	C Total	E Total
I	1 65		.8	2.57	1 66	1 42	44	81	6.50	8.33
II	2.09	1 26	65	2.33	1 33	3 11	? 16	.92	8.33	9.5?
III	1 33	.53	48	.18	0 47	1 04	1 90	1 47	8.52	4.88
IV	1 88	1 50	1 48	1.35	1 48	0.93	1.50	.35	6. 1	6.26
V	3 18	9 40	6.09		1 67	3 75	4 78		18.67	10.20
VI	3 42	3 86	3 81	2.05	3 15	2.97	3.32	3 78	13 14	13 2.
VII	1 65	1 69	1 29	1 63	1 85	0.30	1 68		6.26	3 83

the evening was replaced by 15% w/v ethanol (10 ml/kg) Urine was collected 6–9 p m 9–1 p m 0–6 a.m and 6–12 a.m Urine cAMP was determined by competitive protein binding assay (Linkola & Fyhrquist 1978) Ca and Mg concentrations were measured as described (Ylikahri et al 1974) Hewlett Packard 9810 A calculator was used in paired *t* test analyses.

RESULTS

Urinary excretion of cAMP was primarily suppressed by ethanol in 5 subjects (all but I and VII) (Table 1) In 4 subjects (I II IV and VI) cAMP excretion was stimulated during hangover (6–12 a m) In one subject (I) ethanol only enhanced cAMP excretion Statistically significant effects of ethanol on cAMP excretion were not observed

There was an initial stimulated phase in Ca excretion due to ethanol in most subjects (Table ?) but in some subjects (II and V) Ca excretion was amplified also in later phases of ethanol intoxication Totally ethanol appeared to increase Ca output in 3 subjects (I III and VI) but decrease it in the other four subjects The suppression of urinary Ca output during hangover (0–1? a.m) was almost significant ($t=2$ 176 $P\sim0$ 05) Thus effects of ethanol on Ca excretion appeared to be biphasic

In Mg excretion there was an ethanol-induced stimulated phase in 6 subjects (all but V) (Table 3). Enhanced Mg output appeared to be more widely distributed through the whole measured period in ethanol experiment than enhanced Ca output Totally in 5 subjects (all but IV and V) ethanol increased Mg excretion No statistically significant effect of ethanol was observed

Amplified cAMP excretion was in some cases (II VI and VII) clearly simultaneous with attenuated Ca and Mg excretion and conversely in one subject (III) The symptoms of intoxication and hangover are represented in Table 4 Subjects II III and IV having the lowest urinary Ca excretion and the lowest initial cAMP excretion after ethanol also had nausea and vomitus and the most serious subjective

Table ? *Urinary Ca excreti n in subjects I–VII in mm l during ontr l (C) and ethan l (E) experiment*

	C				E					
	p m		a m		p.m		m			
Subject	6–9	9–12	0–6	6–1	6–9	9–12	0–6	6–1	C Total	E Total
I	0 47		0.81	0.86	0 43	0 79	0 61	0 57	14	2 40
II	0.54	0 35	0.41	0.80	0 59	0 30	0 55	0.62	10	06
III	0.3	0 15	0. 1	0 43	0 47	0 ?9	0 13	0 13	1 0.	1
IV	0.38	0.33	0..5	0 48	0.37	0 48	0 ..	0 11	1 44	1 38
V	1..4	0.52	1 48		1 1	0 3	1 5		3 4	.96
VI	0.5	0.29	0..5	0 43	1 15	0 98	0 35	0 .8	1 49	2. 6
VII	0 86	0 49	0.7?	1 76	0 38	1 03	1 68		3.83	3 09

Table 3 *Urinary Mg excretion in subjects I VII in mmol during control (C) and ethanol (E) experiment*

	C				E					
	p.m.		a.m.		p.m.		a.m.			
Subject	6–9	9–12	0–6	6–12	6–9	9–12	0–6	6–12	C Total	E Total
I	0.8		0.5	0.5	0.5	0.6	0.7	0.6	1.8	2.4
II	1.1	0.7	1.2	1.5	1.1	0.6	1.5	1.4	4.5	4.6
III	0.8	0.7	0.8	0.9	1.3	1.1	0.4	1.1	3.2	3.9
IV	0.7	0.6	0.9	1.0	0.7	0.7	0.5	0.7	3.2	2.6
V	0.9	0.3	1.4		0.8	0.3	1.1		[illegible]	2.2
VI	0.7	0.4	0.6	0.7	1.2	1.4	0.7	0.5	2.4	3.8
VII	0.8	0.9	1.[illegible]	1.3	0.4	1.3	3.0		4.2	4.7

[signs] of hangover Results on blood ethanol, vasopressin, renin, aldosterone cortisol urine output, sodium and potassium were described previously in the same journal (Linkola et al. 1978, Linkola et al. 1979) Subject III having in those previous studies the highest endocrine responses t ethanol also had the highest renal Ca conservation during the control experiment.

DISCUSSION

During the first 2 h of ethanol intoxication cAMP excretion as lower than during the corresponding control time in most subjects (Table 1) This may be due to the suppressed PTH response as has been suggested in rats (Peng & Gitelman 1974) It has recently been reported that during two or six hours ethanol increases serum PTH concentration in rats (Shah et al. 1978), but the corresponding data on man lacking.

In 2 subjects (I and VII) ethanol primarily enhanced cAMP excretion This may be partly attributed to the stimulating effect of ethanol on adenylate cyclase activity observed in many tissues (Volker & Gold 1975) or to increased extrarenal cAMP release Interestingly these subjects also had the lowest endocrine responses to ethanol and no apparent decrease of plasma asopressin after ethanol ingestion (Linkola et al 1978 Linkola et al. 1979) Ethanol reportedly increases serum C (Kalbfleisch et al. 1963 Markkanen & Nantö 1966) This may lead to suppression of PTH response in kidneys (Beck et al. 1974) Thus, high Ca output in subject VII may reflect high renal Ca supply and suppression of cAMP excretion by this ion after 9 p m.

In control experiment urinary excretion of Ca and Mg varied considerably The lowest Ca output was in the subject III having the most pronounced endocrine responses after ethanol, and the highest Ca output was in the subject VII with low endocrine responses to ethanol (Linkola et al 1978 Linkola et al 1979) After ethanol ingestion, all subjects had a phase during which Ca excretion was enhanced when compared to the control experiment (Table) However only in 3 subjects (I III and VI) the total effect of ethanol was stimulative Thus ethanol obviously had a biphasic effect on Ca excretion.

Increased urinary output of Ca has been de scribed n man during ethanol intoxication (Markkanen & Nantö 1966). The present results suggest that this mainly occurs during the early phase of ntoxication. Subjects II III and IV having nausea and vomitus and the most serious subjective signs of hangover (Table 4) had the lowest urinary Ca output during ethanol experiment (Table 2) These subjects also had the lowest cAMP excretion in the

Table 4 *Subjects feeling of hangover and vomiting during ethanol experime t*

Subject	Subjective feeling of hangover	Vomiting during: Intoxication	Vomiting during: Hangover
I	–		
II	+ +	–	
III	+ +		+
IV		–	+
V		+	–
VI		–	
VII		–	–
		–	–

early phase of intoxication The cause or effect relationships of this phenomenon remained unclear However the finding that subject III having the most pronounced endocrine responses to ethanol (Linkola et al 1978 Linkola et al 1979) also during the control experiment had the highest renal Ca conservation may suggest an association between individual responses to ethanol and Ca metabolism Results in subject IV support this assumption Interestingly it has been previously reported that Ca metabolism is associated with ethanol tolerance in rodents because intraventricular $CaCl_2$ enhanced ethanol-induced sleeping-time and behavioral intoxication in rats and mice (Erickson et al 1978) Blood ethanol curves (Linkola et al 1978) indicate that responses to ethanol are hardly due to differences in blood ethanol concentration Moreover subjects II III and IV had blood ethanol peak concentrations later than other subjects which may indicate their slower ethanol absorption rate from the stomach

Similar two phases as in Ca excretion were not seen in Mg excretion after ethanol ingestion In many subjects (all but IV and V) stimulated Mg excretion continued still during hangover Urinary output of Mg and Ca appeared to be at least partly conversely related to cAMP excretion because of the concomitance of low cAMP excretion and stimulated Mg and Ca output This is in agreement with the generally accepted idea that parathormone and cAMP decrease Ca and Mg excretion (Massry & Coburn 1973 Kuntzinger et al 1974) In subject V with the highest cAMP output ethanol was not able to increase Mg output at all On the other hand before the beginning of the experiment this subject exhibited a surprisingly low serum [Mg] (0 70 mmol/l) which may have limited the increase in Mg excretion caused by ethanol (Sullivan et al 1966) In other subjects serum [Mg] was within normal limits and no correlation was observed between serum [Mg] and Mg excretion If the excretion values of Ca regarding this exceptional subject V are not taken into account Ca excretion was stimulated (subjects I III IV VI and VII) or unaffected (subject II) by ethanol during 6–1? p m (paired t-test values was 2 1?0) Also the conservation phase in Ca excretion is more obvious after this manipulation (paired t test value was increased from 2.176 to ? 421 which means significant Ca conservation during 0–12 a.m $P<0.05$)

Hypomagnesemia is reportedly a more frequent and long-lasting consequence of ethanol drinking than other electrolyte changes (Martin et al 1959 It has been suggested causative to ethanol withdrawal syndrome (Victor & Wolfe 197 Hypomagnesemia may also cause tremor hype irritability (Flink et al 1954 Fankushen et al. 196 and decreased response of target organs to PT (Rude et al 1976) Furthermore low Mg levels ha also been suggested to predispose subjects to have severe hangover (Ylikahri et al 1974) Thus th symptoms in subjects II III and IV (Table 4), the low cAMP output and high Mg output in subject may reflect Mg depletion This might also expla why ethanol did not significantly increase M excretion in the present experiment (Sullivan et a 1966) Previously it was already suggested that sub jects II III and IV had lost K (Linkola et al 1978 Thus excessive leakage of intracellular cations M and K may be associated with the symptoms subjects II III and IV The study provides evidenc that Ca and Mg metabolism and the susceptibility the body to the toxic effects of ethanol are interre lated

We thank Mrs Eira Meskanen and Mrs Yrsa Österberg f technical assistance and Mrs Kirsti Alanne for t pe Support was given by the *Finnish Foundation for Alcoh Studies*, the Sigrid Jusélius Foundation the Maen Foundation Helsinki and the Nordisk Insulinfon Copenhagen.

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Acta Physiol Scand 1979 107 339–341

Modification of human pain threshold by specific tactile receptors

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PERTOVAARA, A. Modification of human pain threshold by specific tactile receptors. Acta Physiol Scand 1979 107 339–341 Received 28 April 1979 ISSN 0001-6772. Department of Physiology University of Helsinki, Finland.

The effects of conditioning vibrotactile stimulation of particular tactile receptor groups on thresholds to painful electric stimuli were studied in seven healthy adults. Preferentially Pacinian afferents were activated with conditioning sinusoidal vibration of 240 Hz at 20 and 200 μm amplitudes and preferentially non-Pacinian tactile fibers were activated with conditioning sinusoidal vibration of 20 Hz at 200 and 400 μm amplitudes. None of the subjects showed pain threshold elevation during activation of non-Pacinian tactile fibers. However 6 of the 7 subjects showed significant pain threshold elevation with conditioning vibratory stimulus of 240 Hz at 200 μm amplitude, and 4 subjects showed significantly elevated pain thresholds with conditioning stimulus of 240 Hz at 20 μm amplitude. It is concluded that the activation of Pacinian afferents causes inhibition of pain conducting pathways.

Key words tactile fibers, pain conducting pathways, cross-modal inhibition, cutaneous sensation

Wall & Cronley Dillon (1960) observed an elevation in subjective thresholds for detecting pain in the presence of vibratory stimulus of 60 Hz at an amplitude of 4 mm peak-to-peak. Later it has been shown that the amplitude used at 60 Hz by Wall & Cronley-Dillon activates both Pacinian and intradermal, rapidly adapting receptors (Talbot et al 1968, Merzenich & Harrington 1969). Talbot et al and Merzenich & Harrington showed that high frequency mechanical vibration at low amplitudes activated preferentially Pacinian receptors, whereas low frequency and low amplitude activated preferentially non-Pacinian intradermal receptors.

Ferrington et al (1977) examined human detection thresholds for vibratory stimuli when different tactile receptor groups were simultaneously activated by mechanical vibration of 30 and 300 Hz at low amplitudes. They observed elevations in detection thresholds when preferentially Pacinian receptors were activated by conditioning stimulation. No elevation in detection threshold was observed when selectively non-Pacinian intradermal mechanoreceptors were activated.

The purpose of the present work was to find out whether subjective pain threshold to electric stimuli could be elevated with stimulation of particular tactile receptor groups.

METHODS

Experiments were performed on 7 healthy adults, females and 5 males aged 25 to 40 years. All of them were experienced subjects in physiological testing. During the experiments the subject, who was seated comfortably had his right hand on table, palm downwards.

The sinusoidal mechanical vibration was delivered by an electromechanical vibrator (Bruel & Kjaer Mini-Shaker 4810) driven by function generator (Hewlett-Packard 202C) the signal of which was amplified by conventional power amplifier. Before starting the experiments of this investigation the amplitude calibration of the vibrator was done with miniature piezoelectric accelerometer (Bruel & Kjaer 4339) fixed between the moving coil of the vibrator and the stimulus probe. The diameter of the stimulus probe was 1 cm and it was made of perspex. The dorsal surface of the right hand was stimulated. This area is innervated by the superficial sensory branch of the radial nerve. The stimulus probe was positioned about 1 mm into the skin to ensure constant contact between the stimulus probe and the skin.

The conditioning vibratory stimuli were of 2 frequencies. Firstly 20 Hz sinusoidal at 200 and 400 μm (peak-

Table 1 *The thresholds of one subject to painful electric stimuli without and with each conditioning vibrotactile stimulus and the percentual threshold elevations with simultaneous conditioning vibrotactile stimuli and the statistical significances of the threshold elevations*

The number of measurements was 10 without vibration and 5 with vibration in each condition Ns means nonsignificant

Conditioning stimulus	Pain threshold in mA: Without vibration	With vibration	Percentual elevation	Significance of elevation $P<$
20 Hz, 200 µm	0 10±0 00	0 10±0 00	0 0	Ns
20 Hz 400 µm	0 11±0 02	0 12±0 03	9 1	Ns
40 Hz 20 µm	0 10±0 00	0 14±0.02	40 0	0.001
240 Hz 200 µm	0 12±0 02	0.21±0 06	75 0	0 001

to-peak) amplitudes was used to activate preferentially *non-Pacinian* tactile receptors (anatomically Meissner Merkel and Ruffini receptors) Secondly a sinusoidal vibration of 240 Hz at 20 and 200 µm amplitudes was used to activate Pacinian receptors (Talbot et al 1968, Merzenich & Harrington 1969)

According to threshold estimations on human hairy skin vibratory stimuli of 20 Hz at 200 µm amplitude and of 240 Hz at 20 µm amplitude are roughly of equal subjective magnitude (about 2–3x threshold) and 400 µm at 20 Hz is about 4x threshold whereas 200 µm at 240 Hz is about 20x threshold (Merzenich & Harrington 1969)

Electric pulses of 1 ms duration (with one subject of 8 ms duration) were used as painful stimuli Pulses were applied from Nihon Kohden MSE 3R function generator via Nihon Kohden MSE JM isolation unit to a Grass CCU 1 A constant current unit From the constant current unit the electric pulses were applied to the skin by two closely spaced Pb-Zn cup-electrodes of 8 mm diameter The skin was cleaned with alcohol and the electrodes were smeared with electrode paste The electrodes were placed about 2 4 cm distally from the stimulus probe of the mechanical vibrator Subjects were instructed to report when they first felt a pain that could be described as pinprick The magnitude of the electric current given to the subject could be varied and during a single trial the intensity of current was increased until the subject reported pain It was not possible to begin the trial with suprahminal intensities and then decrease the current because the subjects felt expreme pain only slightly above the pain threshold The pain threshold was determined in 5–10 trials during each conditioning stimulus Prior to the experiment control pain thresholds in mA were determined separately for each subject as an average of 5–10 measurements. During each pain stimulus the threshold in mA was determined three times, once before the vibratory stimulus was started once during vibratory stimulation and once after it For each vibration frequency-amplitude combination 15–25 measurements were made The mean and standard deviation values of each series of trials were calculated and expressed as per cent of the control pain threshold for each individual Student's *t* test (two tailed) was used in statistical calculations.

The duration of the whole experiment was about 15–20 min for each individual and the duration of the threshold determination in each condition was usually about 1 min The order of the conditioning vibrotactile stimuli was in every experiment: 20 µm 240 Hz, 200 µm 20 Hz; 200 µm, 240 Hz, (400 µm 20 Hz was used only with three subjects)

RESULTS

The pain thresholds without conditioning stimuli varied between 0 1 and 1 2 mA and the mean for all subjects was 0 40 mA The range of the standard deviations of the pain thresholds varied between 0 00 and 0 32 mA and the mean of the standard deviations was 0 07 mA

Table 1 gives the results of one subject in different experimental situations and statistical significances of the results

None of the 7 subjects showed significantly elevated pain thresholds during conditioning stimuli of 200 µm and 400 µm at the frequency of 20 Hz 4 subjects showed significantly elevated pain thresholds during conditioning stimuli of 20 µm at the frequency of 240 Hz and 6 subjects showed significantly elevated pain thresholds with conditioning stimuli of 200 µm at 240 Hz.

The mean threshold elevation caused by 20 µm, 240 Hz stimulation was 19% and that caused by 200 µm 240 Hz was 40% (Table 2) The statistical significances of the mean percentual threshold elevations with each conditioning vibrotactile stimulus for all the subjects are also given in Table 2. The mean threshold elevation of all the subjects was significant with conditioning stimuli of 20 and 200 µm at the frequency of 240 Hz.

No after effects were observed the pain thresholds decreased immediately at the termination of the vibration

Pain threshold during simultaneous auditory

Table . Percentual elevations of pain thresholds and their statistical significances for all the subjects

N = non-significant

Conditioning stimulus	The mean threshold elevation, %	Statistical significance, P<
20 Hz, 200 μm	9.4±11.0 (N=7)	N
20 Hz, 400 μm	1.3± 2.3 (N 3)	N
240 Hz, 20 μm	19.0±16.4 (N=7)	0.05
240 Hz, 200 μm	40.1±29.8 (N=7)	0.02

tude of 40 Hz was determined for two subjects and no significant elevation of thresholds was observed.

DISCUSSION

According to previous studies (Talbot et al. 1968, Merzenich & Harrington 1969) 240 Hz vibration activated preferentially Pacinian receptors. And of the two amplitudes used the smaller one (20 μm) probably activated only Pacinian receptors. In the results of Merzenich & Harrington 20 Hz vibration at 200 μm amplitude activated preferentially non-Pacinian tactile fibers, most of which were activated at these amplitudes.

In the present study none of the subjects showed elevated pain thresholds when non-Pacinian tactile afferents were activated. However 6 of the 7 subjects showed threshold elevation when Pacinian afferents were preferentially activated and 4 subjects had higher pain thresholds with high frequency small amplitude vibration which most probably activated only Pacinian corpuscles. Therefore the elevation in pain thresholds with 240 Hz conditioning vibrotactile stimuli was most probably caused by activation of Pacinian afferents. Since activation of non-Pacinian tactile fibers did not elevate pain thresholds it appears that these receptors do not contribute to the pain threshold elevation. However since each sine wave causes one nerve impulse (Talbot et al. 1968) the low frequency vibration used to activate non-Pacinian mecanoreceptive afferents most probably generated less nerve impulses per time unit than the high frequency vibration used to activate Pacinian afferents. Thus it is also possible that the total number of impulses generated in mechanoreceptive afferents contributes to the pain threshold elevation.

In the light of the present experiment the pain threshold elevations caused by 60 Hz vibrotactile stimulus in the experiments of Wall & Cronley Dillon (1960) can be explained as a result of activation of Pacinian afferents. There appears to be similar inhibitory effect from Pacinian afferents to pain-conducting pathways as was assumed to exist from Pacinian afferents to non-Pacinian mechanosensitive pathways by Bystrzycka et al. (1977) and Ferrington et al. (1977).

On the basis of the present experiments it is not possible to exclude the non-Pacinian mechanoreceptive fibers as a source of inhibition to pain-conducting pathways in certain situations e.g. if high amplitude stimulation is used and consequently the number of nerve impulses generated in non-Pacinian afferents is comparable to the number of nerve impulses generated in Pacinian afferents.

I wish to thank Professor J. Hyvärinen for comments on the manuscript.

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Acta Physiol Scand 1979 107 343–363

Firing behaviour in a stochastic nerve membrane model based upon the Hodgkin–Huxley equations

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SKAUGEN E. & WALLØE, L. Firing behaviour in stochastic nerve membrane model based upon the Hodgkin–Huxley equations. Acta Physiol Scand 1979 107 343–363 Received 3 May 1979 ISSN 0001-6772. Institutes of Physiology and Informatics, University of Oslo, Norway

A nerve membrane model with a two-state pore system was investigated by computer simulation in the uniform (space-clamped) case. Both sodium and potassium conducting pores were modelled, each pore having four independent gates which switched randomly between the open and the closed position, governed by the assumed rate constants. Each pore conducted only when all the gates were open. The model was based upon the Hodgkin–Huxley equations for the giant axon in squid, and in the limit of an infinite number of pores it was identical to these. The firing behaviour of this model as function of the number of pores and the injected current were investigated. The mean firing frequency and the distribution of interspike intervals were mainly used in the presentation of the results. It was found that for pore numbers less than about 20 000 the main effects due to a finite number of pores were lowering of the current threshold for firing and a more linear frequency current relationship relative to that of the original H–H equations. For higher pore numbers an increase in the current threshold and pronounced burst firing close to the threshold were found

This is the first of two papers discussing the firing behaviour of a nerve membrane model with a two-state pore system. The model is based upon the Hodgkin–Huxley equations for the squid giant axon membrane (Hodgkin & Huxley 1952)

When Hodgkin and Huxley formulated their equations they discussed a possible physical basis for these. The interpretation the gave as the simplest was that the different ions could only cross a particular region of the membrane when four "particles" were all in a certain position in that region (the open position) Even if only one particle was in the shut position, that region was closed. The total conductance of the membrane at a certain time was then the sum of all the conductances of the open regions of the membrane. The potential dependent parameters α and β in the Hodgkin–Huxley equations then determined the rate of transfer for one type of particle from the "shut" to the "open" and from the "open" to the "shut" position, respectively. This physical interpretation has been strengthened by the discovery that ions cross the membrane mainly through channels or pores and that there is a different pore system for each type of ion (Hille 1970, 1972, 1973). The simplest assumption for the Hodgkin–Huxley equations is then that each pore has 4 "gates" and that an ion can only pass through the pore if it is of the type admitted by the pore, and if all gates are open. The word "gate" is used instead of "particle" in order to make it easier to picture the process.

Very little is known about the processes that open and close the pores. Some experiments on membrane noise seem to indicate that the pores can be partially conducting (Verveen & Derksen 1969, Fishman 1973), but as later results can be well fitted by assuming only an open and a closed state for each pore (Conti et al. 1975, Conti et al. 1976) the former possibility will not be considered here. Other sources of membrane electrical noise are not taken into account here, only the effects of conductance fluctuations are studied.

If we now assume that the gates are independent of each other the α and β parameters in the Hodg-

kin–Huxley equations must determine the probability of transfer for one gate from the "shut" to the "open" and from the "open" to the "shut" position respectively It is then a matter of chance if at a given time a particular pore is open or shut and since the pores are independent the total number of open pores and hence the total membrane conductance will fluctuate around a mean value

The statistical nature of the suggested physical basis of Hodgkin & Huxley's equations was not taken into account when these equations were originally solved the mean values of the different ionic conductances were then used For all practical purposes this is accurate enough when the number of pores is very large as it probably is in the squid giant axon But what happens when the number of pores is small? And how small must the number be for these effects to be important? In order to answer these questions we have simulated a model of the nerve membrane where the statistical properties of the pores were taken into account

This statistical model is meant to represent an isolated patch of nerve membrane for instance a certain length of an axon which is electrically insulated at both ends The patch is so small that there is no need to consider the spatial extension of the nerve membrane and the Hodgkin–Huxley equations can thus be used in their simplest form as a basis for the calculation

The only independent parameters introduced in addition to the parameters in the Hodgkin–Huxley equations are the numbers of sodium and potassium pores in the patch of nerve membrane considered The results are not directly dependent upon the actual area of the membrane but the area will of course determine the total numbers of pores In order to use the results presented here to find the predicted firing behaviour of a space-clamped nerve cell or part thereof one should estimate the number of pores in the membrane and then use this number as the pore number in the figures In most actual cases the membrane will not be described very well by the Hodgkin–Huxley equations and the results presented here must then be taken as indications of the expected effects of a finite pore number This is discussed at the end of the next paper (Skaugen 1979)

Under the assumptions made here the spectra of the membrane conductance fluctuations in the voltage clamped membrane model will be as derived by Hill et al (197?) and Stevens (197?) for the two state pore

One of the most important properties of a nerve cell as an information carrying and processing element in the nervous system is the firing frequency as a function of the input current For the model presented here we have obtained the mean firing frequency and the interspike interval distribution as functions of the injected current and for different values of the number of pores

Stein has made corresponding calculations for a much simpler model of a pore system in the Hodgkin–Huxley nerve membrane model (Stein 1965 1967*a*)

In later papers we shall give corresponding results for a model where the spatial extension of a nerve cell is taken into account. Preliminary reports of the results obtained here in the later papers have been given at meetings of the Scandinavian Physiological Society (Skaugen 1974 1976), and the results have been used in a Ph D thesis (Skaugen 1978)

THEORY

Conventions

The membrane potential is here defined as the potential inside the nerve membrane relative to the potassium equilibrium potential which is accordingly set equal to zero The membrane potential is then always positive, except possibly in the case of an externally induced hyperpolarization This definition makes the computation a little simpler Note that the membrane potential increases if the cell is depolarized decreases if it is hyperpolarized

The equations found by Hodgkin and Huxley for the potential of the squid giant axon (Hodgkin & Huxley 1952) will here be termed the "H–H" equations

Na-conducting pores are termed "Na-pores" and K-conducting pores are termed "K pores"

Assumptions

The model presented here is based upon the following assumptions.

1 There are no potential differences along the nerve membrane

2 The nerve membrane conducts ions through pores. In addition there is a leakage conductance which is constant

3. A particular pore conducts only a certain type of ion, either sodium or potassium ions.

4. All pores which conduct the *same* type of ion have the same conductance when in the open state.

5. There is no coupling between neighbouring gates or pores.

6. Each pore has *four* gates. The pore is open *only if all four gates are open.*

7. The transition probabilities of a gate from one state to another, either from shut to open or from open to shut, are only potential dependent and given by the α and β parameters of the H–H equations.

8. The transition times of the gates are so short that they can be ignored. (The transition time is the time used by the gate to go from the shut to the open position, or vice versa.)

The rest of the assumptions necessary to completely specify the model are stated in the paper by Hodgkin & Huxley (1952). We will restate the necessary equations here.

For a patch of membrane with area A, the membrane potential E is governed by the differential equation

$$\frac{dE}{dt} = -\frac{1}{C}\left(g_{Na}(E-E_{Na}) + g_K E + g_L(E-E_L) - I\right) \qquad (1)$$

where E_{Na} and g_{Na} are the equilibrium potential and the specific membrane conductance for the sodium ions, g_K is the specific potassium conductance (E_K is defined to be zero), and E_L is the equilibrium potential for the specific membrane leakage conductance g_L. C is the specific capacitance of the membrane and I is the specific injected current. With specific values of the parameters we here mean their values per square centimeter of the membrane. Note that the area A does not appear in this equation. The parameters g_{Na} and g_K depend upon the total numbers n_{Na} and n_K of open Na-pores and K-pores

$$g_{Na} = \frac{n_{Na}}{N_{Na}}\bar{g}_{Na} \qquad g_K = \frac{n_K}{N_K}\bar{g}_K \qquad (2)$$

where N_{Na} and N_K are the total numbers of Na-pores and K-pores in the patch of membrane with area A. Instead of total numbers of pores it would perhaps have been more natural to use the specific values of these parameters, that is, the pore densities. But the total numbers are used because we must know these in order *to simulate this model* with a computer. The parameters $\bar{g}_{Na}$ and $\bar{g}_K$ are the specific Na- and K-conductances of the membrane when all the pores are open. In all of the calculations and figures in this paper $\bar{g}_{Na}$ and $\bar{g}_K$ are taken *to be constants equal to their values in squid axon membrane.*

As in the H–H equations the firing behaviour is not directly dependent upon the membrane area A (for the space-clamped case considered here), but it depends upon the numbers N_{Na} and N_K of pores, and it is thus indirectly coupled to this area. Note that the densities (ρ_{Na} and ρ_K) of Na- and K-pores, and the conductances (g_{pNa} and g_{pK}) of single pores now do not enter into these calculations. This *means that we are free to* choose any combination of pore densities and conductances of single pores such that $\bar{g}_{Na} = \rho_{Na} g_{pNa}$ and $\bar{g}_K = \rho_K g_{pK}$ have the values found in the squid giant axon. We are also free to choose any area of the nerve membrane investigated. The total numbers of pores are then given by $N_{Na} = \rho_{Na}A$ and $N_K = \rho_K A$. These numbers are the pore numbers used in the figures.

As an example, let us assume that a space-clamped axon with membrane area $A = 300\ \mu m^2$ is investigated. The sodium pore density ρ_{Na} is taken to be 400 pores per μm^2, so that, as $\bar{g}_{Na}$ is as in squid, g_{pNa} is taken to be $\bar{g}_{Na}/\rho_{Na} = 3\times10^{-10}$ *MHO* per pore. The injected current is 0.018 nA. The total number of Na-pores is now $N_{Na} = \rho_{Na}A = 12\,000$, and the specific injected current is $I = 0.018$ nA/300 $\mu m^2 = 6\ \mu A/cm^2$. These values must then be used in order *to obtain the predicted firing behaviour from* the results presented here.

For the majority of unmyelinated nerve cells the pore densities are generally smaller than assumed in this example, where a realistic value for the squid giant axon was used (Jack 1975). As shown above these smaller pore densities are accounted for by the model presented here, if the conductances of single pores can be assumed to be correspondingly larger. But the conductances of single pores are probably more or less constants, *independent of* the pore densities. That is, the maximal specific conductances $\bar{g}_{Na}$ and $\bar{g}_K$ are smaller than in squid if the pore densities are smaller. The results presented here cannot therefore be applied directly to cases where the pore densities are much smaller than in the giant axon *of squid. This is discussed* in a following *paper*, where it is shown that the results presented here still can be indirectly used as a reasonable approximation (Skaugen 1979).

For the squid giant axon the values of the membrane parameters at 6.3 C are

$\bar{g}_{Na} = 120$ mMHO/cm²
$\bar{g}_{K} = 36$ mMHO/cm²
$g_L = 0.3$ mMHO/cm²
$E_{Na} = 127.0$ mV
$E_L = 22.6$ mV
$E_K = 0$
$C = 1.0\ \mu$F/cm² (3)

According to the H–H equations we must assume that in the Na pores there is one gate of a certain type which we term the h-gate and three gates of another type which we term the m-gate. In the K-pores there are four gates of the same type which we term the n-gate. For the h, m and n gates the probabilities that a shut gate will open in a time interval Δt are $\alpha_h \Delta t$, $\alpha_m \Delta t$ and $\alpha_n \Delta t$ respectively. The probabilities that an open gate will close in a time interval Δt are $\beta_h \Delta t$, $\beta_m \Delta t$ and $\beta_n \Delta t$ respectively. These α and β parameters are functions of the potential only

$$\alpha_h = 0.07\, e^{-0.05E+0.6}\ \mathrm{ms}^{-1}$$
$$\beta_h = (e^{-0.1E\ 4.2}+1)^{-1}\ \mathrm{ms}^{-1}$$
$$\alpha_m = 0.1\,(-E+37)/(e^{-0.1E\ 3.7}-1)\ \mathrm{ms}^{-1}$$
$$\beta_m = 4\, e^{E/18\ 2/3}\ \mathrm{ms}^{-1}$$
$$\alpha_n = 0.01\,(-E+22)/(e^{0.1E\ 2.2}-1)\ \mathrm{ms}^{-1}$$
$$\beta_n = 0.125\, e^{-E/80+0.15}\ \mathrm{ms}^{-1} \quad (4)$$

where E is given in mV relative to E

The assumptions stated above, together with the values of the parameters given here, completely determine the model when the total numbers N_{Na} and N of pores are chosen. It is for instance found that the membrane potential fluctuates around the value $E = 12$ mV when the injected current is set equal to zero.

The model in the limit of an infinite large number of pores

When the number of pores becomes very large the relative fluctuations of the membrane conductances become insignificant. If at a given time the numbers of open h, m and n gates relative to the total numbers of these gates are h, m and n respectively, the expected numbers n_{Na} and n_K of open Na and K pores are close to hm^3N_{Na} and n . Equation (2) then becomes

$$g_{Na} = hm^3\bar{g}_{Na}$$
$$g_K = n^4\bar{g}_K \quad (5)$$

since we assume that in this case the actual parameters are equal to their expected values. Note that h, m and n must have values in the range to 1

In a short time interval Δt the change Δh of h is equal to the relative number of closed gates $(1-h)$ which open minus the relative number of open gates (h) which close in this time interval

$$\Delta h = (1-h)\alpha_h \Delta t - h\beta_h \Delta t \quad (6)$$

Corresponding equations are obtained for m and n. If we divide through with Δt, letting Δt go to zero we obtain

$$\frac{dh}{dt} = \alpha_h(1-h) - \beta_h h$$
$$\frac{dm}{dt} = \alpha_m(1-m) - \beta_m m$$
$$\frac{dn}{dt} = \alpha_n(1-n) - \beta_n n \quad (7)$$

These rates of changes are expected values and must be equal to the actual rates of changes of h, m and n in the limit of an infinite number of pores. Equations (5) and (7), together with Eqs. (1), (3), and (4) are the equations for the giant squid axon membrane found by Hodgkin & Huxley (195?). The model thus reduces to the H–H equations in the limit of an infinite number of pores

The model for a finite number of pores, a simple method of computation

In the case of a finite number of pores a simple method of computation suggests itself. While the computer keeps track of the states of all gates in all the pores, the model time t is increased in small steps Δt. At each step the potential is computed and the α and β parameters are calculated. For each gate which is closed the probability of opening during the next time step is $\alpha\ \Delta t$ and for each open gate the probability of closing is $\beta\ \Delta t$ ($\tau = h$, m or n as required). For each gate the probability is drawn from an appropriate binary probability distribution. The result of the drawing determines if the state of the gate is changed. The total numbers n_{Na} and n of

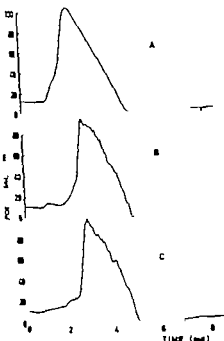


Fig. 1. An action potential calculated by the H–H equations (A), and spontaneously generated (B and C). N =400, and N 100 for B and C, and =0 for all cases. In case A the action potential was started by short current pulse of 0.5 ms duration.

Open pores (where all four gates are open) are then counted and new values of g_{Na} and g are calculated from Eq. (2). To obtain a reasonably accurate calculation Δt must be kept small. The error due to a finite value of Δt can in this and the later methods be evaluated by calculating the mean firing frequency f for different values of Δt taking as the true value the value approached when Δt approaches zero. We will here assume Δt to be sufficiently small if f is less than 2.5 s from the extrapolated true value. This error of 2.5 s is also approximately equal to the standard error of the mean (S.E.) resulting from taking the mean firing frequency f over a model time of 5 s.

Fig. 1B and C shows the potential as function of time calculated by this method for two cases. In both cases the injected current was zero, and the numbers of Na- and K-pores were 400 and 100, respectively. The only difference between the two cases is that they represent different runs on the computer.

The resting potential fluctuates and in both cases it becomes so large at a certain time that an action potential is generated. The membrane thus fires even if the injected current is zero and the H–H equations give a constant potential equal to the resting potential E =1 mV. We note also that the forms of the action potentials thus generated closely follow the form of the action potential calculated by the original H–H equations, which is also shown in Fig. 1.

It is, however, quite impractical to calculate firing frequencies by this method because the computer time used becomes prohibitively large for relatively small pore numbers (larger than 10). And in this case the computer time relative to the model time is proportional to the number of pores, and to the reciprocal of the time step length. In the next section two faster methods for computation are presented.

METHODS

Computation method 2

This method has been used for membranes with total number of pores up to 10 000. The computation time relative to the model time is approximately proportional to the total number of pores, and the accuracy of the method increases as the number of pores increases.

At certain time t_1, the potential E and the state of all the pores are known. It is not necessary to consider the order of open and closed gates of the same type in one pore, the state of pore is therefore here defined by the number of open gates of each type. A Na-pore has 8 possible states and K-pore has 5. These states are for the Na-pore: all gates closed, all m-gates closed and the h-gate open, one m-gate open and the h-gate closed and so on until the state where all gates are open. For the K-pore the states are: all gates closed, one gate open, and so on until all four gates are open. (Here all gates are n-gates.)

The state where k h-gates and j m-gates are open is termed the state SN_{kj}, while the state where j -gates are open is termed the state SK_j. The numbers NN_{kj} and NK_j are the numbers of pores in the states SN_{kj} and SK_j, respectively. The expected values of these numbers are close to

$$NN_{kj} = \binom{3}{j} h^k (1-h)^{1-k} m^j (1-m)^{3-j} N_{Na}$$
$$NK_j = \binom{4}{j} n^j (1-n)^{4-j} N_K \qquad (8)$$

where h, m and can be calculated from Eq. (7). Since α and β_x are functions of E only this calculation is straightforward because the previous values of E are known. In the model, the numbers NN_{kj} and NK_j are integers which fluctuate around their expected values.

The probability that certain pore in the state SN_{kj} or SK_j, will change to the state SN_{pq}, or SK_q, in the infinitesimally small time interval dt is given by

$$P(SN_{kj} \rightarrow SN_{k+1,j})\,dt = \alpha_h\,dt$$
$$P(SN_{kj} \rightarrow SN_{k-1,j})\,dt = \beta_h\,dt$$
$$P(SN_{kj} \rightarrow SN_{k,j+1})\,dt = (3-j)\ \alpha_m\,dt$$
$$P(SN_{kj} \rightarrow SN_{k,j-1})\,dt = j\,\beta_m\,dt$$
$$P(SK_j \rightarrow SK_{j+1})\,dt = (4-j)\,\alpha_n\,dt$$
$$P(SK_j \rightarrow SK_{j-1})\,dt = j\,\beta_n\,dt \tag{9}$$

The time Δt to this change is given by a negative exponential distribution with an expected value $E(\Delta t_1)$ given by

$$E(\Delta t_1) = [P(SN_{kj} \rightarrow SN_{pq})]^{-1}$$
$$\text{or } E(\Delta t_1) = [P(SK_j \rightarrow SK_q)]^{-1} \tag{10}$$

The drawing procedure consists in first drawing the time Δt to the first time one of all the pores changes its state. According to the properties of the negative exponential distribution this time Δt is also given by a negative exponential distribution with an expected value $E(\Delta t)$ given by

$$E(\Delta t) = \left[\sum_{k=0}^{1} \sum_{j=0}^{3} \sum_{\substack{p\ q \\ p\ q\ \pm 1}}^{1} P(SN_{kj} \rightarrow SN_{k+p,\ j+q})\,NN_{kj} + \sum_{j=0}^{4} \sum_{q\ \pm 1} P(SK_j \rightarrow SK_{j+q})\,NK_j \right]^{-1} \tag{11}$$

By a new drawing it is then determined which of the 28 different possible transitions is actualized and the numbers NN_{kj} or NK_j are changed accordingly. This is done by a drawing between each of these 28 possibilities. The probability that just the transition $SN_{kj} \rightarrow SN_{pq}$ will occur for one of the pores in the state SN_{kj} is

$$P(SN_{kj} \rightarrow SN_{pq})\,NN_{kj}\,E(\Delta t) \tag{12}$$

with a corresponding expression for the transition $SK_j \rightarrow SK$. Note that if Eq. (12) is divided by $E(\Delta t)$ we obtain one of the terms in the sum shown by Eq. (11). If for instance the transition $SN_{kj} \rightarrow SN_{pq}$ is drawn, the number NN_{kj} is reduced by one and the number NN_{pq} is increased by one.

The state of the membrane at time $t + \Delta t$ is now known, and the membrane conductances can be calculated from Eq. (2). Note that by definition $n_{Na} = NN_{1,3}$, and $n = NK_4$. The conductances will thus change only if the states SN or SK are involved in the transition. In the time interval Δt from t to $t + \Delta t$ the conductances are always constants and equal to their values calculated at t.

The potential at time $t + \Delta t$ can now be calculated from Eq. (1) because g_{Na} and g are constants in the time interval from t to $t + \Delta t$

$$E = E_\infty + (E - E_\infty)\,e^{-\Delta t/\tau} \tag{13}$$

where $\tau = C/(g_{Na} + g + g_L)$

$E_\infty = (g_{Na}E_{Na} + g\ E + I)\tau/C$

E = value of E at time t

g_{Na} and g are the values of the conductances for $t < t < t + \Delta t$. In a corresponding way new values of h, m and n are calculated by integrating Eq. (7) where α and β are known functions of time since E now is a know[n] function of time. This is not necessary here, but it is us[ed] in some later methods.

At the new time $t + \Delta t$ thus arrived at, the potential and the number of pores in the different states are know[n] and the process described here can be repeated over a[nd] over again, generating E as a function of time.

The only error introduced by this procedure is the u[n]derlying simplification that $E(\Delta t)$ is not dependent up[on] the change of the potential E and hence the parameters α and β in the time interval after t. This is seen by noti[ng] that the transition probabilities as given by Eq. (9) a[re] used in Eq. (11) to calculate $E(\Delta t)$ are calculated at t[he] time t. For large numbers of pores this error is smal[l] because the average time step then is small. This error c[an] be reduced by several methods all of them making use [of] a special property of the negative exponential distributio[n] which is that the probability of an event occurring within [a] small time interval dt is independent of what has happene[d] before. From this it follows that if a time interval Δt [is] drawn which is so long that the potential changes "to[o] much" any point within this time interval can be chose[n] as a starting point for a new drawing.

The simplest method of correcting the error discusse[d] here is to set an upper limit Δt_{max} to the time step of Δt [If] the time step Δt drawn at the time t is larger than Δt_{max} [a] new value of E is calculated at the time $t + \Delta t_{max}$ and th[e] drawing procedure is repeated. The maximum time ste[p] Δt of the calculation of E is thus Δt_{max}. By choosing Δt_{max} sufficiently small, the error in the calculation can be mad[e] as small as we want.

An extra safeguard against too large changes of E in on[e] step of the calculation can be obtained by choosing [an] upper limit ΔE_{max} to the change of E in one step Δt. B[y] using Eq. (13) an upper limit for Δt can in this case b[e] obtained for each new time step

$$\Delta t_{max} = \begin{cases} \Delta t & \text{if } \Delta t \leq \Delta t_E \\ \Delta t_E & \text{if } \Delta t > \Delta t_E \end{cases} \tag{14}$$

$$\Delta t_E = \begin{cases} \infty & \text{if } |E_\infty - E_1| \leq \Delta E_{max} \\ -\tau \ln\left(1 - \dfrac{\Delta E_{max}}{|E_\infty - E_1|}\right) & \text{if } |E_\infty - E_1| > \Delta E_{max} \end{cases}$$

where Δt is the largest allowed value of Δt_{max} and τ i[s] defined in Eq. (13). If Δt is found to be larger than Δt_{max} the drawing procedure is repeated at the time $t + \Delta t_{max}$ a[s] in the simpler method. In the later calculations Δt 0.0[…] ms and $\Delta E_{max} = 1$ mV was used in most cases as this [was] found to give an error in the mean firing frequency f of les[s] than 2.5 s

Computation method 2 b

The method described in the preceding section can be greatly simplified by taking into account only the fluctua[-]tions of the numbers of pores with all m-gates or all n[-] gates open, that is the numbers NN_{m}, NN_{13}, and NK_4. The numbers which are connected to these through the transition probabilities shown in Eq. (9) are NN_{m}, $NN_{\ldots}$

and $NK_{[illegible]}$ and these are then at all times set equal to their expected values given by Eq. (8). In general these numbers are then not integers, but this does not matter because their values are now used only to calculate probabilities. All the other numbers are now of no interest as they can only "influence" the numbers important for the conductance (NN and NK_4) through the numbers $NN_{[illegible]}$, $NN_{[illegible]}$, and NK. This reduces the number of possible transitions from 28 to 8, but the reduction in computer time is even greater. Around the resting potential only about 0.027 and 0.032 of the transitions in the sodium and potassium pore systems, respectively, occur between the states now taken into account.

In order to use Eq. (8) the values of h, m and n must be known at all times when the drawing procedure is started. These parameters must be found by numerical integration of Eq. (7). It is then practical to use the time step length Δt used in the calculation of E. The errors introduced by this numerical integration are then also reduced when the maximum value of Δt is reduced. As the number of pores is increased, the accuracy increases rapidly, and it was found that method 2b could be used for rather low pore numbers, down to 10 pores approximately.

Different combinations of these two methods can now easily be constructed, where all states with 4, 3 and down to j gates open are included in the drawing procedure while the number of states with fewer gates open are set equal to their mean values. This has been done in order to test these methods, but only methods 2a and 2b were used in order to calculate the results presented here. Method 2a was used for N < 10 and $N_{[illegible]}$ < 10, and method 2b was used for the other cases.

When E becomes much larger than E = 12 mV there is a rapid increase of the number of pores in the states where nearly all gates are open, and for method 2b the average length of the time steps Δt decreases correspondingly. The computer therefore uses a disproportionally long time to go through an action potential. To avoid these difficulties this method of computation can be abandoned when E increases over a certain threshold value E_T which seems to be followed by an action potential. (E_T = 40 mV used here.) The expected value of E is then computed from the original H–H equations until the action potential has ended. The numbers of pores in the different states are then set equal to their expected values according to Eq. (8), and the computation method described here is again used. This is a good approximation because the fluctuations of E during and some time after an action potential are small compared to the fluctuations between the action potentials, and the mean firing frequency f is mainly determined by the latter fluctuations. Appendix I shows how the accuracy of method 2b depends upon the number of pores when the action potentials are computed by a nonstochastic method. In the later calculations a threshold of E_T = 40 mV was used for $N_{[illegible]}$ > 100 and N > 100. For smaller pore numbers, method 2b was used during the action potentials also. This gave a negligible error in the mean firing frequency.

The main [illegible] of method 2b is that it of all the changes of states only considers those changes which are important for the number of open pores. This makes it quick. These events are, however, placed accurately, not lumped at certain evenly spaced times as in the simple method described before. This makes it accurate.

This method was found useful for a number of pores up to 10000. For still larger numbers a faster but more inaccurate method had to be devised.

Computation method 3

This method has been used for membranes with a total number of pores from 10000 and upwards. The computation time is independent of the number of pores, and the accuracy of the method increases as the number of pores increases.

The computation is done in time steps Δt of constant length. As in method 2b the fluctuations of $NN_{[illegible]}$, $NN_{[illegible]}$ and NK are neglected and these numbers are set equal to their expected values as shown by Eq. (8). The values of h, m and n are obtained by integration of Eq. (7).

It is convenient for the computation to change the notation, as it is necessary to consider only a small part of the symbols used in method 2a.

$$
\begin{aligned}
S_{[illegible]} &= SN_{[illegible]}, & V_{[illegible]} &= NN_{[illegible]} \\
S_{[illegible]} &= SN_{[illegible]}, & V_{[illegible]} &= NN_{[illegible]} \\
S_{[illegible]} &= SN_{[illegible]}, & V_{[illegible]} &= NN_{[illegible]} \\
S_{[illegible]} &= SN_{[illegible]}, & V_{[illegible]} &= NN_{[illegible]} \\
S_{[illegible]} &= SK_{[illegible]}, & V_{[illegible]} &= NK_{[illegible]} \\
S_{[illegible]} &= SK_{[illegible]}, & V_{[illegible]} &= NK_{[illegible]}
\end{aligned}
\tag{15}
$$

The probabilities p_{ij} that a pore in a certain state S_i will go to another state S_j during the time interval Δt are given approximately by Eq. (9) if Δt is sufficiently small

$$
\begin{aligned}
p_{[illegible]} &= \beta_{[illegible]} \Delta t \\
p_{[illegible]} &= \alpha_{[illegible]} \Delta t \\
p_{[illegible]} = p_{[illegible]} &= 3\beta_{[illegible]} \Delta t \\
p_{[illegible]} = p_{[illegible]} &= \alpha_{[illegible]} \Delta t \\
p_{[illegible]} &= \alpha_{[illegible]} \Delta t \\
p_{[illegible]} &= 4\beta_{[illegible]} \Delta t
\end{aligned}
\tag{16}
$$

and where all other p_{ij} (i, j = 1, 2, …, 6) are zero.

In Eq. (16) the parameters $\alpha_{[illegible]}$ and $\beta_{[illegible]}$ are functions of the potential E. This equation therefore holds true only when E does not change noticeably during the time interval Δt.

The actual number of pores ΔV which goes from a certain state S_i to another state S_j is then given by a binomial distribution, where the probability $p(i, j, k)$ that a certain number k of pores is transferred is

$$p(i, j, k) = \binom{V_{[illegible]}}{k} p_{ij}^k (1 - p_{ij})^{V - k} \tag{17}$$

When the expected value of the number k is large, but small compared to V, this distribution can be approximated by a Gaussian distribution. The net number of pores transferred from state S_j to S_i is described by a corresponding distribution where i and j are interchanged. The net number V of pores transferred is then described by the difference of two Gaussian distributions, which is a new Gaussian distribution

$$\Pr(\Delta V_U \in [k, k+dk]) = \frac{\exp\left\{-\frac{(V_1 p_L - V_1 p_M - k)^2}{2[V_1 p_U(1-p_U) + V_1 p_M(1-p_M)]}\right\}}{\sqrt{2\pi[V\ p_U(1-p_M) + V_1 p_M(1-p_M)]}}\, dk \qquad (18)$$

Note that $\Delta_U = -\Delta V_M$

At each step the numbers ΔV_{31} ΔV_{32} ΔV_{42} and ΔV_{43} are drawn from their respective distributions (ΔV_{ij} is set equal to the integer closest to the result of the drawing) The numbers of pores in the states S S and S are in the time interval Δt changed to

$$V + \Delta V \text{ where } \Delta V = \Delta V_{31} + \Delta V$$
$$V + \Delta V_4 \text{ where } \Delta V_4 = \Delta V_{42} - \Delta V$$
$$V + \Delta V \text{ where } \Delta V = -\Delta V_{43} \qquad (19)$$

The changes occurring in the time interval Δt have thus been calculated but we do not know when these changes occur If we as an approximation assume that all the changes occur at the same time in the interval the calculation becomes particularly simple It is then reasonable to assume that the changes occur in the midst of the time interval Δt Between the times $t - \frac{1}{2}\Delta t$ and $t + \frac{1}{2}\Delta t$ all the conductances are then constants, and in this time interval Eq (1) is easily integrated Since we need to know the value of the potential at time t in order to calculate the changes of the conductances at $t + \frac{1}{2}\Delta t$ the integration is done in two steps

$$E = E_\infty + (E - E_\infty)e^{-\frac{1}{2}\Delta t/\tau}$$
$$E = E_\infty + (E - E_\infty)e^{-\frac{1}{2}\Delta t/\tau} \qquad (20)$$

where τ and E_∞ are given by Eq (13) Note that the conductances in Eq (13) have been calculated at the earlier time $t - \Delta t$ E E and E and the values of the potential at $t - \frac{1}{2}\Delta t$ t and $t + \frac{1}{2}\Delta t$ respectively

We have now arrived at the time $t + \frac{1}{2}\Delta t$ with the potential E and the conductances known The process described here can then be repeated over and over again generating E as a function of time The values of h m and n can then be integrated from Eqs (4) and (7)

This method of computation was also used during the action potentials, since the speed of calculation is, in contrast to method 2*b* independent of the number of open pores

When Δt is decreased there is a decrease of the inaccuracies introduced by the lumping of the conductance changes and the probabilities p_{ij} used At the same time however the expected number of pores transferred from one state to another decreases which decreases the accuracy of Eq (18), and Eq (17) may have to be used In addition the accuracy of this method will decrease when the number of pores decreases, because the fluctuations of the potential then the increase and Eq (13) becomes more inaccurate At the same time the accuracy of Eq (18) also decreases.

Testing the different methods with different step lengths and different pore numbers showed that an error of less than 2.5 s in the mean firing frequency f was obtained for the following restrictions

Method 1	$\Delta t \leq 0.05$ ms
Method 2*a*	$\Delta t_{max} \leq 0.2$ ms
Method 2*b*	$\Delta t_{max} \leq 0.1$ ms and $N \geq 5$ $N_{Na} \geq 1$

Method 2*b* with a non-stochastic calculation of the action potentials.

	$\Delta t_{max} \leq 0.1$ ms and $N \geq 10$ $N_{Na} \geq 1$
Method 3	$\Delta t \leq 0.05$ ms and $N_K = 500$ $N_{Na} \geq 5000$

RESULTS

The internal parameters of the model are determined by the H H equations except for the two independent variables N_{Na} and N_K which are the numbers of Na-pores and K-pores When both of these parameters are infinite the model reduces to the H–H equations We will first study the effects upon the firing behaviour of each of these parameters separately This is done by assuming the other parameter infinite In Figs 2 and 3 it is shown for different values of the injected current how the mean firing frequency over 5 seconds of model time changes as either N_{Na} or N_K is increased from zero towards large values while the other number (N or N_{Na}) is infinite The Appendix shows how the statistical parameters used here are calculated. The values of N_{Na} and N are presented on a logarithmic scale Note that the maximal specific conductances $\bar{g}_{Na}$ and $\bar{g}$ of the pore systems are constants equal to their values in squid axon membrane Eq (3) When the numbers of pores are increased one can interpret this as a corresponding increase of the area of the patch of nerve membrane under consideration

Fig shows that for all values of the injected current used here the mean firing frequency is rather small for low values of N_{Na} especially when N is less than about 10 For low values of N_{Na} ($N_{Na} < 200$) the normal state of the membrane is zero Na-conductance or no Na pores open. This corresponds to the case where $N_{Na} = 0$ which gives a potential E of 11 1 mV above E_K The probability of finding a single Na-pore open at a given time is approximately N_{Na} $hm^3 = 7\ 5 \times 10$ N_{Na} A single Na-pore will occasionally open however This gives an ionic current into the cell of magnitude $(E^N - E)\bar{g}$ = $(13\,800/N^N)\ \mu A/cm^2$ which has the same effect as a sudden increase of the injected current by the same amount According to Hodgkin and Huxley a shock strength of approximately 6 nAs/cm² is barely sufficient to start an action poten-

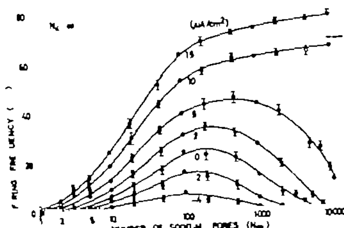


Fig 2 Mean firing frequency as function of N_{Na} for different values of the injected current when N is infinite. Numbers show value of in μA/cm^2. S.E. is shown by ertical bars

l from the resting potential of 1 mV (Hodgkin and Huxley 1952). Using this we find that at $E=$ 2 mV here the mean open time is 0 083 ms, the opening of a single Na-pore gives a mean shock strength of 6 nAs/cm^2 if $N = 190$ For lower values of N the occasional opening of a single Na-pore ll in most cases start an action potential The rapid increase of the mean firing frequency f when N increases towards 190 therefore reflects the increasing rate of openings of single Na-pores At the same time the probability that two or more Na-pores open at about the same time begins to become significant. E en if the opening of single pore fails to start spike, the potential is increased somewhat, and this increases the probability that a second pore will open. The increasing rate of multipore openings also contributes to the increase of the mean firing frequency f which reaches a maximum at about 200 Na-pores for $=0$ As N^* increases above 190, however the opening of a single Na-pore more and more often fails to start an action potential Moreover the fluctuations of the Na-conductance due to the random opening and shutting of Na-pores eventually start to decrease and f decreases to zero as N^* is increased further

When the injected current is increased, the mean value of the potential is also increased and this increases the probability that a Na-pore will open This event is now also more likely to tart an action potential. The mean firing frequency f is thus increased at all values of N_{Na}. The local maximum of f around N_{Na} 200 for $I=0$ is shifted towards higher values of N_{Na} when is increased, due to the smaller current shock necessary to start a spike. At the same time multipore openings begin to become important at lower values of N_{Na}, due to the increased probability of pore opening.

When the injected current I is smaller than the threshold current $_T=6.3$ μA/cm^2 in the H–H equations the mean firing frequency f decreases to zero as N_{Na} is increased. When I is larger than I the asymptotic value of f for $N_{Na}=\infty$ is indicated by broken lines to the extreme right in the figure

The effects of a finite number of K-pores are shown in Fig. 3 When there are no K-pores ($N =0$) the K-conductance is zero and there is a stable value of the potential E for $E>E = 22.6$ mV when the injected current I is larger than zero. Repetitive firing is not found due to the lack of the K-conductance. With a single K-pore the situation is different. During the peak of an action potential the probability that the single K pore will open is very high and this must eventually happen. The potential is then rapidly reduced towards zero and the probability that the open K-pore will close increases.

When this happens the potential first increases towards the equilibrium potential E of the g conductance which is then dominating. This increases the Na-conductance and a new action potential is generated. When there are several K pores the same process is repeated when the last K-pore closes after an action potential a new spike is triggered off The mean firing frequency is reduced towards zero when the injected current is decreased

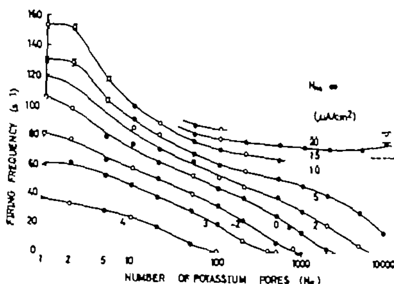


Fig 3 Mean firing frequency as a function of N_K for different values of the injected current I when N_{Na} is infinite Numbers show value of I in $\mu A/cm^2$ S.E. is shown by vertical bars

from zero towards $-4.7\ \mu A/cm^2$ At this value the closing of all K pores simultaneously will not be sufficient to start an action potential Note that there is no corresponding lower limit of the injected current for firing when we have a small number of Na-pores

When N_K is increased multi-pore closing and opening become more important but the mean firing frequency decreases If the injected current I is smaller than the current threshold I_T the mean firing frequency f eventually falls to zero When I is larger than I_T the asymptotic value of f when N increases is larger than zero and is indicated by the broken lines to the extreme right in the figure In this case the examples used in the figure when $I=10$ 15 and 20 $\mu A/cm^2$ show that a minimum value of f is obtained for $N \approx 1000$ When N_K is increased above this value there is a slight increase of the firing frequency

The data used in Figs. 2 and 3 can also be presented as the mean firing frequency as a function of the injected current for different values of N_{Na} and

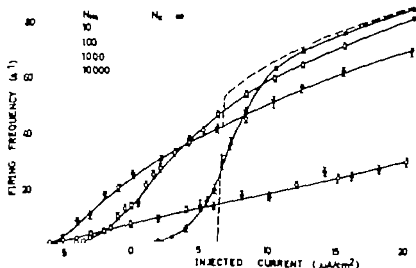


Fig 4 Mean firing frequency as a function of the injected current for different values of N_{Na} when N is infinite. Broken line shows the case where also N_{Na} is infinite S.E. is shown by vertical bars.

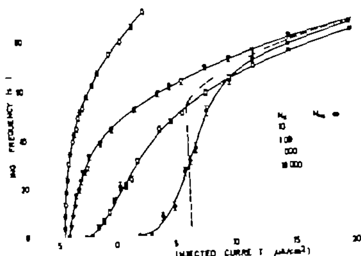


Fig. 5. Mean firing frequency as function of the injected current for different values of N when N_{Na} is infinite. Broken lines show the case where also N is infinite. S.E. is shown by vertical bars.

Y as shown by Figs. 4 and 5. The corresponding curve for an infinite number of both Na- and K-pores is shown by the broken line (Stein 1967*b*). The most noticeable effects on this current-frequency relationship introduced by a finite number of either Na- or K-pores are a change of the current threshold for firing, and a much more linear relationship. This is similar to the results obtained by Stein in his calculations (Stein 1967). But in his model the behaviour of the pores was assumed independent of the membrane potential and the pores were assumed to carry all of the current. Twenty open pores were assumed to give the threshold current and their mean open time was taken to be 1 msec. The pores were also assumed to give positive currents inwards, and would thus correspond to sodium pores. As each of the "pores" in Stein's model would have about 12 times as large an effect upon the membrane potential as the sodium pores assumed here due to the longer mean open time, 1 msec against 0.083 msec, his model would correspond roughly to our model with $N_{Na}=(E_{Na}-E)$ $/(I_T/20$ pores (1 msec/0.083 msec))≈3 600 pores. The results presented in his

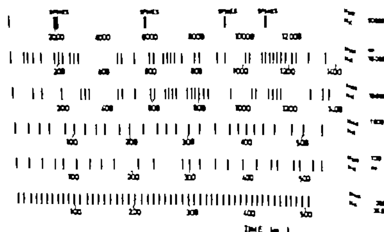


Fig. 6. Trains of action potentials for different values of N_{Na}, N and the injected current (in $\mu A/cm^2$). The values are shown in the figure. Note changes of time scale.

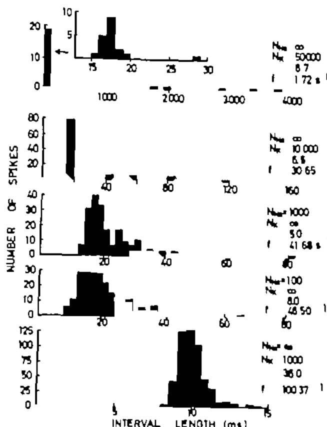


Fig 7 Interspike interval histograms for different values of N_{Na}, N_K and the injected current i (in $\mu A/cm^2$). The values are shown in the figure. These examples are also used in Fig 6. Note changes of interval length scale.

Fig. 7 then agree well with the results obtained here as shown in our Fig 4.

The minimum of f around $N_K = 1\,000$ which was seen in Fig 3 is more clearly shown in Fig 5 where we see that for $i \geq 9$ $\mu A/cm^2$, $N_K = 1\,000$ gives a lower firing frequency than both higher and lower values of N_K.

At pore numbers of 10, 100, 1 000 and 10 000 the results of Figs 2 and 4 and 3 and 5 can be cross-checked. This gives the smaller uncertainties in Figs 2 and 3 at these numbers.

When the number of pores is increased above 5 000 pores, the distribution of spikes in a train of action potentials changes markedly. Fig 6 shows typical spike trains for pore numbers from 100 to 50 000. For pore number below 5 000 the spikes in the train seem to be randomly distributed, but as the pore numbers are increased above this value there is an increasing tendency of the spikes to occur in bursts. For large numbers the typical burst behaviour is fairly long trains of action potentials with a regular firing frequency of approximately 55–60 s⁻¹ separated by long periods with no firings. The length of these silent periods fluctuates greatly.

This burst activity is due to a larger probability for firing about 20 ms after the peak of an action potential. This is connected with the slight overshoot of the membrane potential over its final value about 20 ms after an action potential, which is seen in the H–H equations (Hodgkin & Huxley 1952), in the case of a single firing. For small pore numbers, when the fluctuations are large compared to this overshoot, it has almost no effect. But for large pore numbers, when the fluctuations are small compared to the overshoot, there is a greatly increased probability for firing at this time. If the membrane fails to fire then, however, it can take very long time before the fluctuations by chance build up sufficiently to trigger off an action potential. In the original H–H equations it is seen that for an injected current i close to the threshold for firing, the spikes occur just at this overshoot. If the membrane fails to fire then (because i is too small) it cannot fire at a later time unless i is increased. This explains the minimum firing frequency $f_T = 55$ s⁻¹ of the H–H equations (Hodgkin & Huxley 1952, Stein 1967*b*). The time from a spike to this overshoot is just equal to $1/f_T$. Accordingly, the mean firing frequency during bursts of spikes should be approximately equal to f_T, which fits well with the results from the stochastic models.

The minimum firing frequency $f_T = 55$ s⁻¹ in the H–H equations is important in the following discussion of the results obtained, and is termed the threshold frequency. It is the point of division between quite different modes of behaviour in the stochastic models.

Fig 7 shows examples of the interspike interval histograms for finite numbers of either the Na- or the K pores, and for different values of the injected current i. These histograms show the effects of the stochastic fluctuations of the nerve membrane conductances upon the distribution of the interspike intervals. These effects are quite similar for the two cases of either a finite number of Na pores or a finite number of K pores. No systematic differences between these two cases could be seen by inspection of the histograms which were obtained during the computations.

When there is typical burst firing, the peak of the histogram is narrowed and shifted to the left relative to the base, because most of the interspike intervals are now of approximately the same short length $1/f$

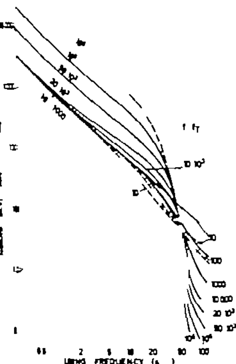


Fig. 8. Standard deviation ΔT of the interspike intervals from their mean value $1/f$ as a function of the mean firing frequency f for different values of N_{Na} and N. The numbers shown are the values of N_{Na} when N is infinite (continuous lines), and of N when N_{Na} is infinite (broken lines, shown only if different from the other case). Dotted lines show the relations $\Delta T = 0.8/f$ and $f = f$

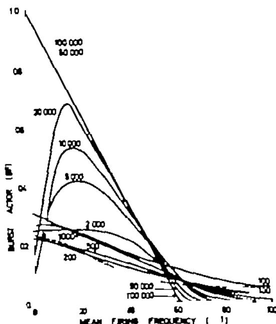


Fig. 9. Burst factor BF as a function of the mean firing frequency for different values of N_{Na} when N is infinite (continuous lines), and of N when N_{Na} is infinite (broken lines, shown only if different from continuous lines). Dotted line shows simplified relationship given by Eq. (1.4) with $f = 60$ s

burst intervals), but some intervals are much longer than these (the intervals during bursts). In order to get a quantitative expression for the degree of burst firing, a burst factor BF is defined as

$$BF = 1 - \frac{\text{Median of the interval}}{\text{Mean of the intervals}} \quad (21)$$

With a symmetrical distribution the median is equal to the mean and the burst factor BF = 0. When there is typical burst activity with almost all intervals equal and short, and a few intervals very long, the mean will be much larger than the median, and BF approaches one. Note that BF is a measure of the skewness of the interspike interval distribution.

Fig. 7 shows that the width of the histograms changes a lot when the injected current and the pore numbers are changed. The standard deviation ΔT of the intervals from their mean, as calculated in the Appendix is here used as a measure of this width.

Figs. 8 and 9 show the parameters ΔT and BF respectively as functions of the mean firing frequency f for different pore numbers. Note that for BF linear axes are used, while logarithmic axes are used for ΔT. It was found much more useful to present ΔT and BF as functions of f than as functions of the injected current. Among other things this made it easier to compare the firing behaviour for different pore numbers. When N_{Na}, N and f are specified, I is given and cannot be chosen independently. Its value can be obtained from Figs. 2, 3, 4 and 5.

Each of the curves shown in Figs. 8 and 9 is drawn as the best fit to the results obtained. The different symbols shown are used only to distinguish the different curves. An example of the data obtained in the actual simulations is shown in Fig. 10, where the curves used in Figs. 8 and 9 are also shown. For pore numbers above 200 the results were almost independent of the types of pores considered, and in these cases only one curve is used to

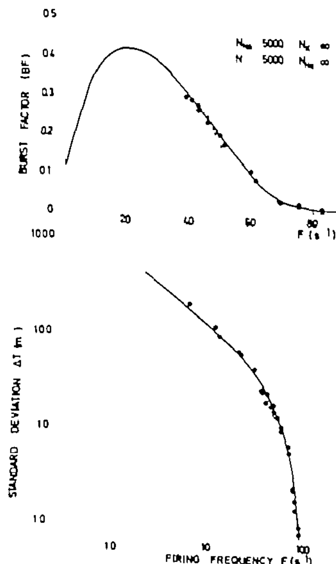


Fig. 10. Results from simulation which were used to obtain curves similar to those shown in Figs. 8 and 9 for $N_{Na}=5000$ and N infinite (filled circles) and for $N=5000$ and N_{Na} infinite (open circles).

show the results for N_{Na} infinite and N equal to the number indicated and for N_K infinite and N_{Na} equal to the same number.

When the mean firing frequency f was less than f_T and for pore numbers larger than about 100000 the burst firing became so pronounced that it was not possible to obtain reliable estimates of ΔT (or BF) because the burst periods and the intervals between them often became longer than the maximum model time which could be simulated. (This time was set equal to 70 s for economical reasons.) But a minimum value of ΔT could be obtained. The model was then "sampled" several times and minimum values of the mean number of spikes in a burst were estimated at different values of the injected current. Minimum values of ΔT were then calculated according to the simple model in the Appendix.

It should be noted that for small values of f there is a large uncertainty in the results shown by Figs. 8 and 9 because the data are then based upon a few interspike intervals. This is seen as a spreading of the results as f is decreased.

Fig. 8 shows that for firing frequencies below f all the curves for pore numbers less than 50000 are close to each other independent of the number and types of pores. For mean firing frequencies f below about 70 s⁻¹ these curves can be approximately described by a straight line given by $\Delta T=0.8/f$. For all pore numbers the curves are well approximated by straight lines described by ΔT=constant/f for $f<70$ s⁻¹ (except for 70000 pores). In the Appendix it is shown that for burst firing ΔT will for $f \ll f_B \simeq 60$ s⁻¹ be given by $\Delta T=\sqrt{2N_B+1}/f$. Eq. (1.9). Fig. 8 indicates that N_B, the mean number of intervals in one burst, is a function of the pore number only, independent of f for $f<70$ s⁻¹. The constant $\sqrt{2N_B+1}$ can be read directly from the figure as the value of ΔT in seconds for $f=1$ s⁻¹. This gives that N_B is 4, 17 and 70 for pore numbers of 50000, 10⁵ and 10⁶ respectively. For pore numbers smaller than 20000 $\Delta T \cdot f=0.8$, which indicates that the variability is not mainly due to burst activity (since from Eq. (1.9) N_B then is negative). These results agree well with the burst behaviour of the spike trains (see Fig. 6 for some examples).

For mean firing frequencies f above f_T the standard deviation ΔT continues to decrease when f is increased. But here this decrease is much more rapid for large pore numbers than for small pore numbers. As the pore numbers are increased the case of a regular firing is approached, and in the limit of infinite values of both N_{Na} and N the interspike intervals are all equal, which gives $\Delta T=0$, as in the non-stochastic H-H equations.

The results shown here are similar to the results obtained by Stein with his pore model (Stein 1967*a*), which corresponds to our model with 3600 Na pores. The data presented in his Fig. 9, where he shows $\Delta T/\bar{t}$ as a function of $1/f$, closely matches the results obtained here for $1000<N_{Na}<10000$.

When f is smaller than f_T, however, there is no underlying regular firing and the durations of the interspike intervals fluctuate even if N_{Na} and N approach infinite values. Fig. 8 shows that ΔT even increases when the number of pores is increased

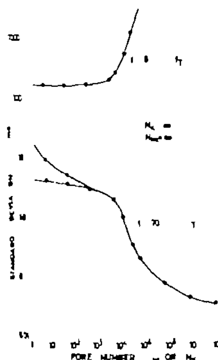


Fig. 11 Standard deviation ΔT as a function of N_{Na} when [illegible] (continuous lines), and of N when N_{Na} is [illegible] (broken lines, shown only when different from continuous lines) and for f 5 and $f=70$

[illegible] 20 000 in this case This increase is due to the increasing burst activity when the pore numbers are decreased, in particular the increasing number of spikes in one burst The results shown by Fig. 8 are presented in a more condensed form in Fig. 11 which shows ΔT as function of the pore numbers for f 5 $^{-1}$ and $f=70$ s

It should be noted that there do not exist any stable firing frequencies below f for the H–H equations (Stein 1967*b*) The behaviour of these equations cannot therefore be properly approached by increasing the pore numbers if f is kept in the region between 0 and f For any finite pore numbers it is possible to keep f in this region by a sufficiently careful adjustment of the injected current As we see from Figs 4 and 5 the current region which gives non-zero firing frequencies less than f decreases when the number of pores is increased, but for finite pore numbers it will not become zero

The burst factor BF shown in Fig. 9 as a function of the mean firing frequency f is seen to approach the relationship given by Eq. (1 4) when the number of pores is increased. This relationship is shown by the dotted line in the figure where a burst frequency $f=60$ s has been chosen The mean firing frequency during the bursts actually changes slightly when the mean firing frequency f is changed but for large pore numbers this change is almost unnoticeable in the figure. For pore numbers larger than 50 000 the relationship given by Eq (1 4) is closely followed If the pore numbers are decreased from this value a large deviation is first seen at small values of f For pore numbers of 20 000 for instance there was no clear burst activity when f was smaller than 10 s The increase of BF above the theoretical relationship (dotted line) when the pore number is decreased is due to the increasing fluctuations of the length of the interspike intervals during bursts ($f<f_B$) or during continued firing ($f \gg f_B$).

The results shown here are presented in a more condensed form in Fig. 12, which shows BF as a function of the pore numbers for $f=25$ s and $f=70$ s

Fig. 13 shows the current threshold I for firing as a function of the pore numbers N_{Na} and N In the stochastic model investigated here, there is no sharply defined threshold for firing as in the H–H equations, where the threshold current is $I_{T0}=6.3$ μA/cm^2 (shown by the dotted line in the figure) For pore numbers less than 50 000, I is here defined as the value of the injected current I which gives a mean firing frequency of $f=1$ s This value is found from figures similar to Figs. 4 and 5 The vertical bars shown in the figure indicate the variation of I obtained by drawing the curves in Figs. 4 and 5 at the extremes of the vertical bars shown there

For large pore numbers, could not be reliably estimated by this method due to the pronounced burst firing. At small values of f the intervals between bursts often became longer than the maximum model time which could be simulated and a reliable estimate of f could not be obtained. Instead I was defined as the value of I which, with a probability of 0.5 gave firing up to one second after the onset of the current. At the onset the membrane almost always responded with at least one action potential which often was the start of a typical burst of spikes. If the burst ended and no new burst was initiated before the second period ended, the injected current used was defined as below threshold If the firing continued out the one second period, or if new burst, or even a single spike, was

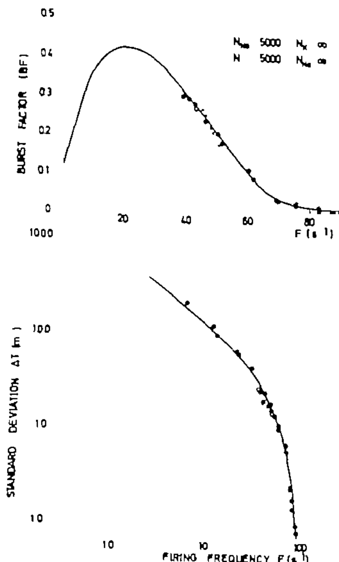


Fig 10 Results from simulations which were used to obtain curves similar to those shown in Figs. 8 and 9 for N_{Na}=5 000 and N infinite (filled circles) and for N =5 000 and N_{Na} infinite (open circles)

show the results for N_{Na} infinite and N equal to the number indicated and for N_K infinite and N_{Na} equal to the same number

When the mean firing frequency f was less than f_T and for pore numbers larger than about 100 000 the burst firing became so pronounced that it was not possible to obtain reliable estimates of ΔT (or BF) because the burst periods and the intervals between them often became longer than the maximum model time which could be simulated (This time was set equal to 20 s for economical reasons) But a minimum value of ΔT could be obtained The model was then "sampled" several times and minimum values of the mean number of spikes in a burst were estimated at different values of the injected current Minimum values of ΔT were the calculated according to the simple model in th Appendix

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For mean firing frequencies f above f_T the standard deviation ΔT continues to decrease when f is increased But here this decrease is much more rapid for large pore numbers than for small pore numbers As the pore numbers are increased the case of a regular firing is approached and in the limit of infinite values of both N_{Na} and N the interspike intervals are all equal which gives ΔT=0, as in the non-stochastic H–H equations

The results shown here are similar to the results obtained by Stein with his pore model (Stein 1967*a*) which corresponds to our model with 3600 Na pores The data presented in his Fig. 9 where he shows $\Delta T/f$ as a function of 1/f closely matches the result obtained here for 1 000<N_{Na}<10 000

When f is smaller than f however there is no underlying regular firing and the durations of the interspike intervals fluctuate even if N_{Na} and N approach infinite values Fig. 8 show that ΔT even increases when the number of pores is increased

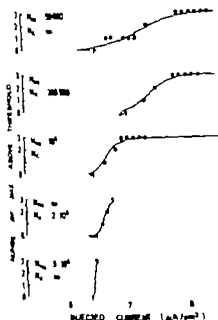


Fig. 14. Number of cases above threshold as function of the injected current for different values of N_{Na} and N. These values are shown in the figure. Examples of results used is shown for pore numbers larger than 10 000. See text for explanation.

pore number is increased. A correspondingly larger value of is then needed to give these fluctuations a possibility to start an action potential. The value $_{T0}$ is reached by at N_{Na} or N ≈40 000 and a maximum value of 7.6 $\mu A/cm^2$ is reached at about 100 000 pores. As the pore number is further increased, decreases towards I_{T0}. The reason for the increase of I above $_{T0}$ is closely connected to the mechanisms which give burst firing. For large pore numbers (>20 000) the conductance fluctuations are very small and do not affect the generation of an action potential much. If the injected current is slightly above $_{T0}$, the model therefore fires quite regularly with firing frequency approximately equal to that of the H–H equations (f≈f). This is the behaviour during a burst of spikes. The conductance fluctuations may, however, delay a spike so much that the period for greatest probability for firing is passed, and the firing then stops. The model is now in situation which corresponds to the one obtained in the H–H equations by slowly increasing to its present value. Due to accommodation the firing will not then start even if is somewhat higher than I_{T0}. When the firing in the model stops, a relatively large fluctuation is therefore needed to start the firing again and this may take a long time to happen. This silent period is the period between bursts. When the current I is increased the probability that the firing will start again increases while the probability that it will stop decreases. For small pore numbers it is the probability that the firing starts which is important while for large pore numbers (>100 000) it is the probability that the firing stops which determines the threshold.

We will now consider the case where both N_{Na} and N are finite. Figs 15 and 16 show the mean firing frequency f as functions of N_{Na}/N when the total number $N_{Na}+N$ of pores is 500 and 2 000, respectively. The numbers N_{Na} and N must then change between zero and the sum $N_{Na}+N$ when N_{Na}/N is increased from zero to infinity. The dotted line shows f as a function of N_{Na} when N is infinite and the broken line shows f as a function of N when N_{Na} is infinite. These curves are taken directly from Figs 3 and 4. In most cases the fluctuations of the numbers of open Na- and K-pores combine to give a firing frequency which is higher than at least one of the values of f obtained from each of these pore systems separately. We cannot expect a linear addition of these effects, because the stochastic processes described by the model are highly nonlinear. For small values of N_{Na} (N_{Na}<45 in Fig. 10), the combined effects of the finite Na- and K-pores even give a smaller value of f than the effect of either the system of Na-pores or the K-pores alone. At these low values of N_{Na} the probability that a Na-pore will open is very small even if all K-pores close and the potential increases to E =12.6 mV. And when the Na-pore system in this way does not respond to sudden increases of the potential no action potentials will be generated.

The firing behaviour was further investigated for a constant value of the quotient N_{Na}/N equal to 4. This is a rather arbitrary choice; the only reason it was preferred being that it gives approximately equal conductances for single Na- and K-pores. Fig. 17 shows the mean firing frequency f as a function of the injected current for different values of the total number $N_{Na}+N_K$ of pores. It is seen that the general form of the frequency current relationship lies between those of the two cases considered before, that is, either N_{Na} infinite or N infinite.

The other phenomena seen, burst firing and the

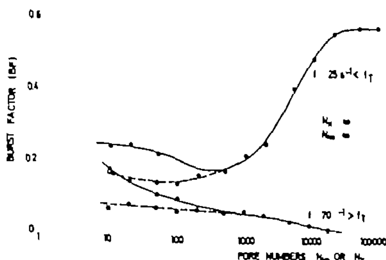


Fig 12 Burst factor BF as a function of N_{Na} when N is infinite (continuous lines), and of N when N_{Na} is infinite (broken lines, shown only when different from continuous lines) and for $f = 25$ s and $f = 70$ s

initiated during the one second period I was defined as above threshold Note that one spike generated per second gives $f = 1$ s the threshold value used for small pore numbers

Around the value of I where the current threshold was believed to be found three simulations were performed for each value of I which were changed in steps of 0 1 $\mu A/cm^2$ Fig 14 shows some typical results obtained The number of times a result was obtained which indicated "above threshold" is plotted for each value of I If normalized the curve fitted to these results gives the probability of obtaining firing up to one second after the onset of the current as defined above The threshold current I_T is now found as the value of I where this curve gives a probability of 0 5 and this value of I is plotted in Fig 13 (For N_{Na} or $N_K \geq 70000$.) The vertical bars in Fig 13 show the current range where the probability for firing lies between 0 1 and 0 9

Fig 13 shows that the threshold current I_T increases towards the threshold current I_{T0} in the H–H equations when the pore number is increased from small values This is due to the decreasing amplitude of the conductance fluctuations when the

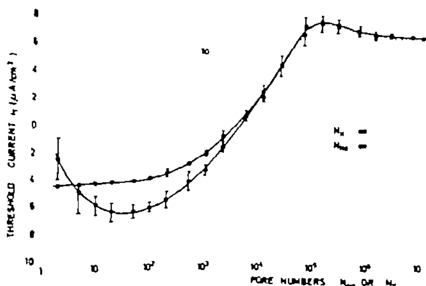


Fig 13 Threshold current I for firing as function of N_{Na} when N is infinite (filled circles) and of N when N_{Na} is infinite (open circles) Uncertainty shown by vertical bars See text for explanation.

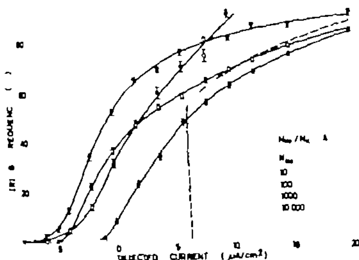


Fig. 17 Mean firing frequency as a function of the injected current for different values of $N_{Na}+N_K$ when $N_{Na}/N_K = 4$. Broken line shows the case where $N_{Na}+N_K$ is infinite. S.E. is shown by vertical bars.

(*a*) a different current threshold for firing, smaller for small pore numbers and larger for large pore numbers (>40 000).

(*b*) a more linear relationship between the mean firing frequency and the injected current.

(*c*) fluctuations of the length of the time intervals between two action potentials (the interspike intervals).

(*d*) burst firing for large pore numbers (>2 000).

These differences become unnoticeable when the number of pores approaches infinity but they were clearly seen for surprisingly large pore numbers up to several hundred thousand for case ( ).

It is worth noting that for large pore numbers (N_{Na} and $N_K \geq 5000$) the firing behaviour for a certain number of Na-pores ($N_K = \infty$), is almost identical to that of the same number of K pores ($N_{Na} = \infty$) even if the gating mechanisms are quite different in the two cases.

The change of the threshold could easily have been obtained in the original H–H equations by for instance a change of the leakage conductance g. The more linear relationship is, however much more functionally important for the cell. It may for instance mean larger capacity to transmit information. This result is made even more interesting by the fact that it is difficult to obtain an approximately linear current-frequency relationship by equations of the same type as the H–H equations. It can be done, however by fine tuning of the α and β parameters and by including some additional membrane conductances (Skaugen 1975 Connor & Stevens 1971).

DISCUSSION

The calculations presented here were made in order to find how the firing behaviour of a well-known, space-clamped nerve membrane depends upon the number of sodium and potassium conducting pores. The Hodgkin–Huxley equations were chosen as an example, but corresponding results would be expected if another set of equations had been chosen, for instance the equations describing a node of a myelinated nerve fibre in toad (Frankenhaeuser & Huxley 1964). That is, one would expect a lowering of the firing threshold, a more linear frequency-current relationship, etc as the total number of pores was decreased (by decreasing the total membrane area for instance). The phenomenon of burst firing is, however closely connected with the maximum probability for firing at a certain time after the action potential (~20 ms for the H–H equations) but occurs only very close to the threshold current for firing. In the H–H equations the virtual discontinuity of the frequency-current relationship around the threshold is also due to this phenomenon. In nerve cell models where the non-stochastic equations do not show this discontinuity (as the model for the isolated Gastropod neurone soma (Connor &

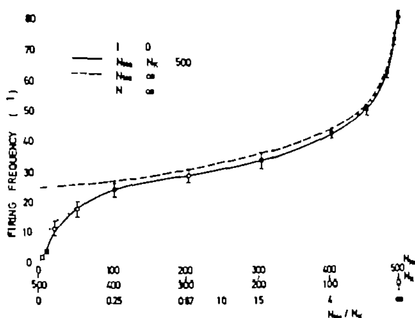


Fig. 15. Mean firing frequency f as a function of N_{Na}/N_K when $N_{Na}+N_K = 500$ (continuous line). Dotted line shows f as a function of N_{Na} when N_K is infinite and broken line shows f as a function of N_K when N_{Na} is infinite. S.E. is shown by vertical bars.

change of threshold were quite similar to the results obtained for the two cases considered before, that is N_{Na} finite and N_K infinite and vice versa. This is as expected since these two cases gave approximately the same results when the standard deviation ΔT, the burst factor BF and the current threshold for firing I_T were considered.

Conclusion

The results presented here show that when the effects of a finite number of pores are introduced in the H–H equations the firing behaviour of the nerve membrane can be quite different from that predicted by the original H–H equations. These differences are mainly

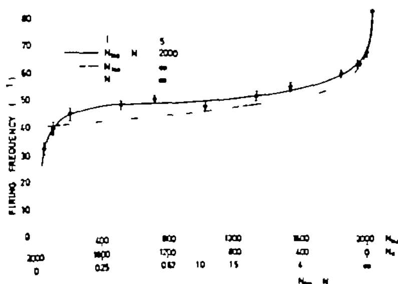


Fig. 16. Mean firing frequency f as a function of N_{Na}/N_K when $N_{Na}+N_K = 2000$ (continuous line). Dotted line shows f as a function of N_{Na} when N_K is infinite and broken line shows f as a function of N_K when N_{Na} is infinite. S.E. is shown by vertical bars.

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Stevens 1971) we would therefore not expect so pronounced burst firing for large pore numbers as in the H–H equations

A general discussion is presented at the end of the following paper where effects of gating currents multistate pore systems and other sources of electrical noise in the nerve membrane are considered (Skaugen in press) It is also shown that from simple space-clamped membrane models like the one discussed here it may be possible to obtain some general indications of the effects of finite pore numbers upon the firing behaviour of physiologically operating nerve cells

We would like to thank Daniel Kernell David Attwell Julian J B Jack Jan K S Jansen Knut Liestøl Arild Njå and Sveinung Rekaa for stimulating discussions and comments on the manuscript and Torhild Isachsen for drawing the figures. The work was supported by The Norwegian Research Council for Science and the Humanities

APPENDIX CALCULATIONS OF SOME STATISTICAL PARAMETERS

Results of the simulations are presented for the case where the injected current is zero up to time zero and then is increased suddenly to a constant value. This is the value of the current shown in the figures. The mean firing frequency is calculated by

$$f = N \Big/ \sum_{j=1}^{N} T_j \qquad (1.1)$$

where the T_j s are the interspike time intervals and N is the number of these

An estimate of the standard deviation ΔT of the interspike intervals from their mean value was obtained by the following expression

$$\Delta T = \left(\frac{1}{N-1} \sum_{j=1}^{N} (T - 1/f)^2 \right)^{\frac{1}{2}} \qquad (1.2)$$

where $T_{N+1} = T + 1/f$—time at last spike

From Eq (1.2) an estimate of the standard error of the mean of f can be obtained

$$\Delta f = \frac{1}{2}\left[\left(\frac{1}{f} - \frac{\Delta T}{\sqrt{N_1}}\right) - \left(\frac{1}{f} + \frac{\Delta T}{\sqrt{N}}\right)\right] \qquad (1.3)$$

In the figures the interval $f-\Delta f$ to $f+\Delta f$ is shown by vertical bar

In the typical burst activity a burst of $N+1$ spikes (N intervals) is followed by a silent period of duration T. For different bursts both N and T change a lot but the firing frequency f during a burst is approximately constant, and the fluctuations of the interspike intervals during a burst can be neglected. If the mean value of N is larger than one the median of the interspike interval distribution is then $1/f_B$ while the mean of this distribution is $1/f$ where f is the mean firing frequency. According to Eq (1.1) the burst factor BF is then given by

$$BF = \begin{cases} 1 - f/f & \text{for } f < f_B \\ 0 & \text{for } f \geq f \end{cases} \qquad (1.4)$$

In general f is a function of f. f increases somewhat when f is increased but f_B will always be close to the threshold frequency f when $f \ll f$. The simulations showed that f was nearly constant in this case with a value $f \approx 60$ s. The burst factor BF will then be described by two straight lines

Assume now that in the period of observation a number of N bursts and N silent periods of length T_k occur. If N is the mean value of N_B, the standard deviation ΔT from the mean of the interspike intervals can be determined from Eq (1.2)

$$\Delta T^2 = \frac{N\,N\,(1/f - 1/f_B)^2 + \sum_{k=1}^{N} (1/f - T_k)^2}{N(N+1)} \qquad (1.5)$$

The mean value T of T can be expressed by the other parameters used here

$$T_m = \frac{1}{N} \sum_{=1}^{N} T = \frac{1}{f} + (N+1)\left(\frac{1}{f} - \frac{1}{f}\right) \qquad (1.6)$$

By using Eqs (1.5) and (1.6) ΔT can then be written as

$$\Delta T = \left(N_m \left(\frac{1}{f} - \frac{1}{f}\right) + \frac{T^2}{N+1} \right)^{\frac{1}{2}} \qquad (1.7)$$

where $T^2 = \frac{1}{N} \sum_{k=1}^{N} (T - T)$

The long interburst intervals T may be assumed exponentially distributed for $t > 1/f$ if the T are large compared to the correlation time for correlations in the membrane potential [13]. T is then equal to $T - 1/f_B$, since all T are larger than $1/f$ and Eq (1.7) becomes

$$\Delta T = \left(\frac{1}{f} \quad \frac{1}{f}\right) \sqrt{2N+1} \qquad (1.8)$$

For the case where f is small this simplifies to

$$\Delta T\, f = \sqrt{2N_m + 1} \quad f \ll f_B \qquad (1.9)$$

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On the nature of basal vascular tone in cat skeletal muscle and its dependence on transmural pressure stimuli

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GRÄNDE, P-O BORGSTRÖM, P & MELLANDER S On the nature of basal vascular tone in cat skeletal muscle and its dependence on transmural pressure stimuli. Acta Physiol Scand 1979 107 365–376. Received 17 May 1979 ISSN 0001-6772. Department of Physiology and Biophysics, University of Lund, Sweden.

The aim of the present study was to elucidate in some detail the characteristics of the intrinsic basal vascular tone in the adrenergically blocked skeletal muscle with regard to its extent and site along the vascular bed its dependence on arterial pressure in static and dynamic transmural pressure stimuli and its sensitivity to local metabolic influence. Basal tone, which apparently is of myogenic nature, was pronounced in 'proximal arterial vessels' (>25 µm i.d.) and in the 'microvessels' (<25 µm), but low in large veins Its functional characteristics, however were different in the 'proximal arterial vessels' and the microvessels Normal basal tone in the microvessels thus seemed to be intimately dependent on the arterial blood pressure level and, at least partly initiated by its static mean pressure distension effect as well as by its dynamic pulse pressure oscillations It could be virtually abolished by transmural pressure decrease applied at fast rate ('strong inhibitory dynamic transmural pressure stimulus') Basal tone in the proximal arterial vessels on the other hand, was little affected by arterial pressure and almost unresponsive to transmural pressure stimuli Basal tone in the 'microvessels' was much more sensitive to metabolic stimuli than that in the 'proximal arterial vessels' The present results, viewed in the light of some recent electrophysiological studies on vascular smooth muscle suggest that smooth muscle in the microvessel is mainly of the spike-generating type, whereas that in the proximal arterial vessels seems to be of different nature, possibly of the non-spike-generating type.

Key words Basal vascular tone myogenic mechanisms, transmural pressure stimuli, pulse pressure autoregulation, metabolic control spike-generated tone

In 1902 Bayliss put forward his classical hypothesis that the intravascular pressure via distension caused a direct stimulating action on the vascular smooth muscle contributing to existing vascular tone and its regulation The term myogenic reactivity has since then by convention been used to denote the ability of the vascular smooth muscle to respond to the mechanical force exerted by blood pressure terminology which is adopted in the present paper Although the myogenic hypothesis of Bayliss was questioned for considerable length of time (e.g. by Anrep 1912, and others) it received quite conclusive experimental support from more recent hemodynamic and vital microscopy studies (e.g. Folkow 1949, Folkow & Öberg 1961 Mellander Öberg & Odelram 1964 Johnson 1968 Baez, Laidlaw & Orkin 1974).

The myogenic mechanism is thus now quite widely appreciated as an important factor in the development of active responses to changes in arterial blood pressure for instance in autoregulation of blood flow (for ref see Johnson 1964) implying that it acts by increasing or decreasing the normal basal vascular tone Basal vascular tone in this context refers to the marked intrinsic tone which at normal pressure remains on the arterial side of most vascular beds after elimination of known nervous and humoral excitatory influences. There is much to indicate that this basal tone is mainly of myogenic origin (e.g. Folkow 1962) but

its initiating mechanisms are not yet experimentally defined Basal tone might possibly result from spontaneous myogenic automaticity in the vascular smooth muscle but it might also require some specific continuing stimulus for its development and maintenance for instance initiated by the arterial blood pressure distension as apparently envisaged by Bayliss It is possible that basal tone might be established by somewhat different mechanisms in the various sections of the vascular bed in view of the documented heterogeneity of vascular smooth muscle in different parts of the circulatory system (e g Bohr 1965 Vanhoutte 1978)

In the present investigation an attempt was made to elucidate some characteristics of the basal tone in consecutive sections of the vascular bed of skeletal muscle special attention being paid to its dependence on vascular transmural pressure stimuli. The fact that the myogenic response to decreased transmural pressure is effected by inhibitory interference with basal tone might imply that myogenic reactions and myogenic basal tone are mediated via some common cellular mechanism(s) Further insight into this problem might be gained from more detailed studies about such myogenic inhibitory interaction particularly in view of a recent description of the cellular mechanisms behind myogenic reactivity in single unit vascular smooth muscle in vitro (Johansson & Mellander 1975)

Since similar mechanisms might be operating in vivo as well it would be of interest to determine the maximal extent to which basal vascular tone in different sections of the vascular bed can be inhibited by a true myogenic reaction evoked by decrease in transmural pressure Recent experiments (Grande et al 1977 1978) have demonstrated the existence of a small static and a much more prominent dynamic or rate sensitive component in myogenic vascular control in skeletal muscle the former component related to the amplitude of the steady state constant increase or decrease in vascular transmural pressure (P_T) and the latter to the rate of the transmural pressure change (dP_T/dt) Observations of the active dynamic myogenic dilator response to a strong inhibitory (negative) dP_T/dt stimulus in terms of a rapid transmural pressure decline were therefore made when applied at normal basal tone Comparison of this active dilator response with that evoked by a supramaximal dose of papaverine (which abolishes vascular tone whatever its cause) provided a rough estimate of the maximal extent to which basal tone could be inhibited by myogenic mechanisms evoked via transmural pressure stimuli.

In a second series of experiments various attempts were made to determine to what extent basal tone in different sections of the vascular bed could be dependent upon the normal arterial blood pressure level Special attention was paid to the possibility that the arterial pressure via static and/or dynamic transmural pressure stimuli could contribute to the initiation and maintenance of basal myogenic tone

The results indicate that the basal tone is of somewhat different nature in the microvessels compared to that in the more proximal arterial vessels. This difference will be tentatively discussed in the light of some recent electrophysiological studies on vascular smooth muscle.

METHODS

The study was performed on 25 cats b wt. 3.–4 3 kg, anesthetized with α-chloralose (50 mg/kg b wt.) and urethane (100 mg/kg b wt.) The cats were breathing spontaneously through a tracheal cannula. Body temperature was kept at 38±0 5°C A few preparations which were technically unsuccessful were excluded from the study

Experimental preparation The observations were made on the α- and β-adrenergically blocked vascular bed in the acutely denervated lower leg muscles of the right hindlimb The muscle region was prepared so that the popliteal artery and vein formed the sole vascular connections with the body Surgical procedures and the experimental set up were basically the same as described previously (see Grände Lundvall & Mellander 1977) and will be only briefly summarized The muscle region was autoperfused via a shunt circuit placed between the femoral and the popliteal artery except for one series of expts. where the muscle region was perfused with a pressure servo-controlled roller pump (see below). Mean arterial blood flow (Q) to the region was continuously recorded with a reliable pressure gradient flowmeter (Grande & Borgstrom 1978) Venous blood flow from the region as diverted via a catheter to the right jugular vein Mean arterial inflow pressure (AP) and venous outflow pressure (VP) were measured from T-tubes close to the cannulated popliteal artery and vein By means of a screw clamp placed around the shunt tube arterial pressure could be adjusted to desired level Pressures from arterial microvessel (small artery pressure SAP) and from venous microvessels (small vein pressure SVP) were continuously monitored using a technique modified from that originally described by Haddy et al (1954) (for detail about this technique and its validity see Grande 1979) For this purpose the sural artery and vein on the posterior surface of the gastrocnemius muscle were ligated at a site just distal to the adjacent fat pad and flexible nylon tubings (d of tip ~0 3 mm) were inserted just below

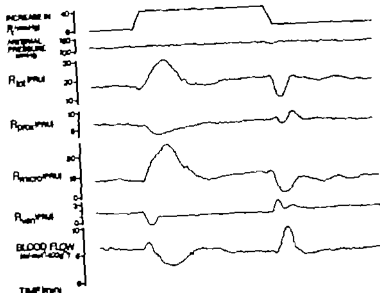


Fig. 1. Effects of strong dynamic transmural pressure (P_T) stimuli ($dP_T/dt = \pm 5$ mmHg/s) on basal vascular tone in cat skeletal muscle. Note the marked reinforcement of microvascular basal tone (R_{micro}) evoked by the positive dynamic stimulus, and, especially, the almost complete abolition of this tone evoked by the negative dynamic stimulus.

tree ligatures. Pressures were recorded with Statham transducers. All parameters were registered on a Grass Polygraph.

The muscle preparation with the intact vascular connections was placed in a hermetically closed temperature-controlled plethysmograph (38°C) filled with Tyrode' solution. The fluid in the plethysmograph was connected via tubing with an external, open reservoir mobile in the vertical direction to permit variation of the hydrostatic fluid pressure in the plethysmograph to desired levels in relation to atmospheric pressure (Grande, Hirlsch & Mellander (1974). In the control situation (i.e. at atmospheric plethysmographic pressure), venous pressure was adjusted to level (=5 mmHg) which established an isovolumetric state of the preparation and kept at this level throughout the expt. By lowering the reservoir all parts of the muscle vascular bed could be exposed to increased vascular transmural pressure (P_T) and this could be applied at any desired rate dP_T/dt.

In one series of expts somato-motor nerve stimulation was instituted and accomplished by electrical stimulation (3 V, 1 ms) of the distal end of the cut sciatic nerve after adrenergic and cholinergic blockade.

The following substances were used. Heparin (1 000 I.U. kg⁻¹ b.w.), phenoxybenzamine (10 mg kg⁻¹ tissue), propranolol chloride (1 mg kg⁻¹ tissue), and atropine sulphate (1 mg kg⁻¹ tissue). The latter substances were given i.a. and caused effective autonomic blockade of the vascular bed.

Data are presented as mean values ± S.E.

Recordings of vascular resistance. With the use of additional pressure transducers (National Semic. LX 1601 D) and electronic divider circuits, as described by Grande & Borgström (1978), the present technique permitted continuous recordings of total and segmental resistances in the muscle vascular bed according to the following definitions. Total vascular resistance = (AP–VP)/Q; proximal arterial resistance = (AP–SAP)/Q; microvascular resistance = (SAP–SVP)/Q and large vein resistance (SVP–VP)/Q. This approach, according to previous evaluations (see Grände et al. 1977, 1978, Grände 1979), permits reliable recordings of the mentioned vascular resistances. This holds true also during phasic change of vascular transmural pressure, except for 'large vein resistance' which then is somewhat unreliable due to the induced transcapillary fluid loss.

Pump perfusion expts. In one series of expts shifts were made from normal pulsatile to non-pulsatile pressure perfusion. For this purpose a pressure servo-controlled roller pump was inserted in the arterial shunt circuit. This pump could simulate normal cardiac pulsations by feeding into the servo-control system a reference input signal of normal undamped arterial pressure obtained from a pressure transducer connected to the brachial artery. The pump permitted shift to non-pulsatile perfusion at unchanged mean arterial inflow pressure.

RESULTS

The expts were performed on the sympathectomized and adrenergically blocked vascular bed of skeletal muscle with well preserved basal vascular

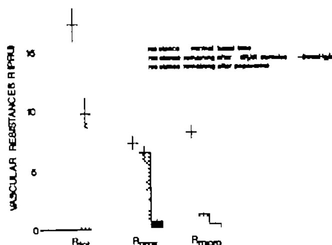


Fig 2 Average effects ($n=8$) of a strong inhibitory dynamic transmural pressure stimulus ($dP_T/dt = -5$ mmHg/s) and of a supramaximal dose of papaverine on basal tone in the whole vascular bed (R_{tot}) in the proximal arterial vessels (R_{prox}) and in the microvessels (R_{micro}). Note the pronounced inhibition of basal tone by the dynamic transmural pressure stimulus effected by an almost selective relaxation in the microvessels

tone within the normal range (cf Mellander & Johansson 1968) Total and segmental vascular resistances in the muscle region were followed continuously throughout the expt from blood flow and differential pressure measurements (see Methods) The average value for small artery pressure in the present material was 70±3 mmHg at an arterial inflow pressure of about 125 mmHg indicating that small artery pressure was monitored from arterial microvessels smaller than about 25 μm (i d) (cf Fronek & Zweifach 1975 Grände 1979) The site for separation along the vascular tree between the proximal arterial vessels and microvessels therefore roughly refers to this vessel dimension.

Effects of a strong inhibitory transmural pressure stimulus on basal vascular tone As mentioned in introduction the myogenically reactive vascular smooth muscle is especially sensitive to dynamic transmural pressure stimuli dP_T/dt Previous studies (Grände & Mellander 1978) have indicated that such a stimulus with regard to inhibitory responses approaches its effector maximum (dilation) at a dP_T/dt value of -5 mmHg/s which was applied in this study and accomplished by a P_T decrease of 40 mmHg in 8 s To minimize the inevitable (and disturbing) passive vascular collapse effect especially in the venous section of such a P_T decline the expts. were performed as illustrated in Fig. 1 The vascular bed was first exposed to a P_T increase of 40 mmHg above normal control, in this case at the rate of 5 mmHg/s accomplished by a decrease of extravascular (plethysmographic) pressure P_T was then maintained at this constant increased level until the pronounced dynamic constrictor response had vanished and the small static constrictor response had reached a steady state After this the test inhibitory transmural pressure stimulus was applied by decreasing P_T back to the control level at the specified rate of -5 mmHg/s. The initial P_T increase thus served the dual purpose to minimize passive vascular collapse and to prevent vascular transmural pressure from falling below the normal control level during the subsequent P_T decline

The figure shows recordings of the resistance responses evoked in the whole vascular bed (R_{tot}) in the proximal arterial vessels (R_{prox}) in the microvessels (R_{micro}) and in the large veins (R_{v}) to such transmural pressure stimuli. The responses to the mentioned P_T increase confirm in essential respects our previous observations (Grände et al 1977). It evoked a strong dynamic myogenic constriction in the microvessels in response to phasic increase in P_T i e. to the positive dP_T/dt stimulus which was reflected in total vascular resistance also whereas the proximal arterial vessels and especially the veins were almost irresponsive to the dynamic stimulus merely showing passive distension The static myogenic constrictor responses to constant increased P_T were small in the microvessels comprising about 2.5 PRU and in the proximal arterial vessels about 0 8 PRU above the control values Large vein resistance was simultaneously decreased passively slightly below control The net effect on overall vascular resistance to constant increased P_T was moderate as depicted in the R_{tot}-curve

The vascular responses to the inhibitory dP_T/dt stimulus of -5 mmHg/s are shown in the latter part of Fig 1 Note the marked active dilation in the microvessels which lowered microvascular resistance from about 10.5 PRU to 1.5 PRU thus far below the initial control value of about 8.5 PRU at normal basal tone (see initial part of the curve). The proximal arterial vessels showed a bi-phasic response an initial active dilation which lowered resistance somewhat below the initial control level followed by moderately raised resistance A small resistance increase also was noted in the large vein section Increased resistance in the latter sec

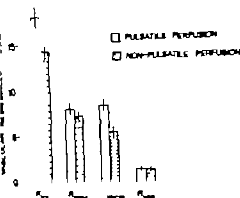


Fig. 3. Effects of shift from pulsatile to non-pulsatile perfusion at constant mean perfusion pressure on total and segmental vascular resistances in skeletal muscle. Note the marked decline in total vascular resistance (R_{tot}) during non-pulsatile perfusion, an effect mainly caused by inhibition of basal tone in the microvessels. Resistance in the proximal arterial vessels (R_{prox}) was much less, and in 'large' veins (R_{ven}) hardly at all affected.

tions during the P decline most likely can be ascribed to passive elastic recoil (which thus could not be prevented entirely). The described active dilator responses, however dominated the behaviour with regard to overall vascular resistance resulting in a net decrease in R_{tot} from the control value of about 18 down to 10 PRU.

The described pattern of response to the strong inhibitory transmural pressure stimulus was similar in all expts (8 cats). For the whole material (see Fig.), this stimulus decreased microvascular resistance from an average control value at normal basal tone of 8. ±0.6 PRU down to an average of 1.3 0. PRU and proximal arterial resistance (during the initial dilator phase) from 7.3±0.5 to 6.5 0.4. Total vascular resistance decreased from an average of 17.4±1.4 to 9.9±0.8 PRU. These dilator effects should be compared to those (n=8) evoked by a supramaximal dose of papaverine (100 mg/kg muscle tissue) which caused complete elimination of the vascular smooth muscle tone. Microvascular resistance during such papaverine infusion averaged 0.4 0.1 PRU proximal arterial resistance 0.7±0.1 PRU 'large' vein resistance 0.5 0.1 PRU and total vascular resistance 1.7±0.2 PRU at normal perfusion pressure.

The data in Fig. show that a pronounced basal tone existed in the vascular bed under control conditions which could be abolished by papaverine and that this basal tone mainly resided in the proximal arterial vessels and the microvessels whereas the 'large veins' had low tone. The applied transmural pressure stimulus (negative dP_T/dt) was capable to very effectively inhibit basal tone in the microvessels where resistance decreased by almost 90% of that evoked by papaverine but was less effective in the more proximal arterial vessels where resistance during the initial dilator phase decreased by only some 10% of the papaverine effect. Data for this inhibitory effect in the large vein section are not included in the figure since they are less reliable due to hazards in determining resistance in these vessels during phasic shift in P_T where also a passive elastic recoil effect was quite significant. Mention should be made that some passive elastic recoil might have been present in the other vascular sections as well especially in the proximal arterial vessels being quite distensible (cf Caro et al. 1978), which implies that the inhibitory effects of the dP_T/dt stimulus might have been somewhat underestimated by the data given in Fig. . A rough estimate of this effect was obtained by determining it on the papaverine-treated vascular bed in response to the negative dP/dt stimulus. Such analysis suggested that the mentioned effect was relatively small.

The fact that the applied dynamic inhibitory transmural pressure stimulus via a myogenic mechanism was capable to effectively interfere with basal tone only in the microvessels suggests that basal tone in the microvessels and in the larger arterial vessels is of somewhat different nature.

Effects of normal arterial pressure pulsations on basal vascular tone. Some previous studies (Mellander & Arvidsson 1974, LaLone et al. 1975) have shown that shift from normal pulsatile to non-pulsatile arterial pressure perfusion leads to decreased overall vascular resistance in a skeletal muscle preparation, suggesting that the arterial pulsations might contribute to the establishment of normal basal vascular tone, perhaps via repetitive dynamic transmural pressure stimulation. The aim of this series of expts. was to investigate if such an effect is mainly confined to the microvessels, a possibility suggested by the results in the previous section showing that basal tone in these vessels was especially sensitive to the experimentally applied external dynamic transmural pressure stimulus.

For this purpose total and segmental vascular

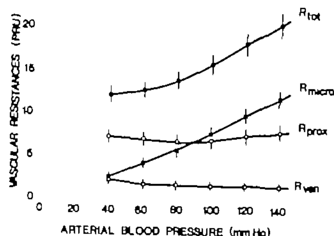


Fig. 4. Effects of short term (<60 s) reductions of arterial blood pressure on total and segmental vascular resistances in skeletal muscle. Note that the evoked decline in total vascular resistance could be almost entirely explained by dilation in the microvessels.

resistances in the adrenergically blocked muscle region were analysed at normal basal tone upon shift from pulsatile to non-pulsatile perfusion accomplished with the use of a servo-controlled roller pump (see Methods) under maintenance of a normal and constant mean arterio venous pressure difference.

Fig. 3 summarizes the average result of 8 observations on 5 cats. It can be seen that shift from pulsatile to non-pulsatile perfusion caused a pronounced inhibition of basal tone in terms of a decrease by almost 20% in total vascular resistance in the muscle region. Note that the major part of this dilator response was localized to the microvessels (smaller than about 25 μm i.d.), the resistance of which decreased by almost 35% below control (or about 35% of the maximal papaverine induced inhibitory response). Some inhibition of tone occurred also in the more proximal arterial vessels in which resistance decreased by some 10%. A special test expt. on the papaverine-relaxed vascular bed demonstrated that total and segmental vascular resistances, if anything, were somewhat smaller during pulsatile than non-pulsatile perfusion. This indicates that the resistance decline during non pulsatile perfusion, as depicted in Fig. 3, was caused by active smooth muscle responses and not by passive effects, for instance related to rheological or other physical phenomena (cf. Mellander & Arvidsson 1974).

The demonstrated clear inhibition of normal basal tone upon elimination of the arterial pulsations at maintained normal mean distending pressure thus seems to be an effect mainly localized to the microcirculation. Such oscillations thus appear to be essential for the development of normal microvascular basal tone and the effect might result from repetitive positive dynamic transmural pressure stimuli on the vascular smooth muscle evoked during the rising phase of the pulse pressure curve.

Effects of arterial pressure reduction on basal vascular tone and possible underlying mechanisms. Observations of total and segmental vascular resistances in the adrenergically blocked muscle region were performed on 5 cats during graded reductions of arterial inflow pressure which was accomplished by adjustments of a screw-clamp placed around the arterial shunt tubing (see Methods) proximal to the site of the arterial pressure measurement. Such compression of the tubing produced not only a decrease of mean arterial blood pressure from its control value of about 140 mmHg in these expts. but also a simultaneous gradual attenuation of the arterial pulse pressure oscillations which were virtually abolished at a mean pressure of 40 mmHg. Mean arterial pressure was reduced to the various levels depicted on the abscissa in Fig. 4 and this was done for short periods of time (<60 s) to minimize possible metabolic vasodilator influence. The resistance data on the ordinate in Fig. 4 refer to the steady state after such a short-lasting pressure reduction. Complete recovery of vascular tone was instituted between successive pressure reductions.

It can be seen from the figure that total vascular resistance in the muscle region (R_{tot}) decreased with decreasing arterial inflow pressure. This resistance decline was steepest in the pressure range down to about 80 mmHg and implied a fairly well developed autoregulation of blood flow in this pressure range. Note that the decline in total vascular resistance could be almost entirely explained by dilation in the microvessels, the basal tone of which was almost completely abolished (inhibited by almost 80% of maximum) at the arterial pressure of 40 mmHg. Resistance in the proximal arterial vessels and the large veins remained fairly constant or at the low arterial pressure levels even tended to increase with decreasing arterial pressure. The latter effect might mainly be ascribed to passive elastic recoil in these quite distensible vessels (cf. Caro et al. 1978) during the reduced arterial pressure which thus possibly could have obscured some minor concomitant inhibition of their basal tone.

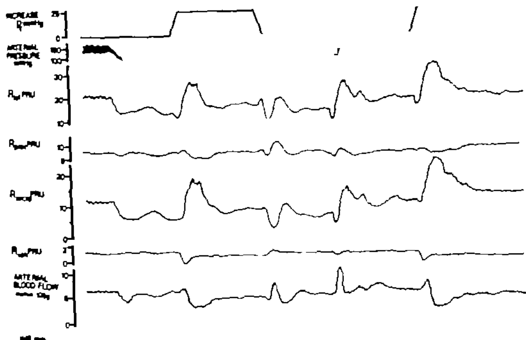


Fig. 5. Experiment serving to illustrate that basal tone in the microvessels (R_{micro}), which is markedly decreased by reduction of arterial pressure, can be partially restituted by experimental restoration of mean distending pressure to normal accomplished by superimposed increase of transmural pressure (P) via decrease of extravascular pressure. The experiment also demonstrates that dynamic and static myogenic reactivity is quite well preserved during the induced arterial hypotension. For further details see text.

It is concluded that basal vascular tone in the microvessels is distinctly dependent upon the arterial pressure level and an attempt will be made to elucidate the underlying mechanisms. It can be deduced from the data in Fig. 3 that a significant part of the described inhibitory effect on micro vascular tone of arterial pressure reduction can be attributed to the concomitant experimental attenuation of the arterial pulsations. Some 35% of the inhibitory effect could be ascribed to the latter factor at the lowest arterial pressure level (40 mmHg), where the pulsations were virtually eliminated. The remaining inhibitory effect might be attributed to several factors, for instance to decreased static transmural pressure distension caused by fall of mean arterial pressure to decreased dynamic transmural pressure stimuli (see Discussion) to possible metabolic inhibitory influence on basal tone and/or on myogenic reactivity etc.

The expt. depicted in Fig. 5 provides some information about the importance of static transmural pressure distension for vascular tone and about static and dynamic myogenic reactivity during arterial pressure reduction. In this expt. arterial pressure was reduced from about 150 to 100 mmHg i.e. in the range where blood flow was reasonably well autoregulated to minimize possible metabolic dilator influence. Flow (mean value) was thus reduced by only some 1 % in this case. Total vascular resistance (R_{tot}) decreased by about 5.5 PRU during the pressure reduction, almost entirely explained by inhibition of basal tone in the microvessels.

This arterial pressure reduction led to a fall of mean distending pressure in the whole vascular bed by about 25 mmHg, calculated as (AP+VP)/2 and to attenuation of the pulse pressure oscillations. The low mean distending pressure could now under maintained arterial hypotension be roughly restored to normal by increasing overall vascular transmural pressure via decrease of plethysmographic (extravascular) pressure by the same amount, a procedure shown in Fig. 5. This compensatory increase of transmural pressure was applied at the rate of 25 mmHg/ and led to an initial pronounced dynamic myogenic constrictor response in the microvessels which raised vascular

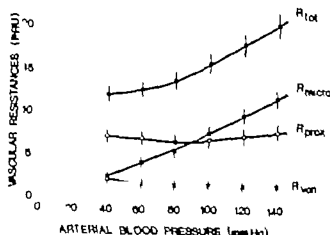


Fig 4 Effects of short term (<60 s) reductions of arterial blood pressure on total and segmental vascular resistances in skeletal muscle Note that the evoked decline in total vascular resistance could be almost entirely explained by dilation in the microvessel

resistances in the adrenergically blocked muscle region were analysed at normal basal tone upon shift from pulsatile to non-pulsatile perfusion accomplished with the use of a servo-controlled roller pump (see Methods) under maintenance of a normal and constant mean arterio-venous pressure difference

Fig 3 summarizes the average result of 8 observations on 5 cats It can be seen that shift from pulsatile to non pulsatile perfusion caused a pronounced inhibition of basal tone in terms of a decrease by almost 0% in total vascular resistance in the muscle region Note that the major part of this dilator response was localized to the microvessels (smaller than about 25 µm i d) the resistance of which decreased by almost 35% below control (or about 35% of the maximal papaverine-induced inhibitory response) Some inhibition of tone occurred also in the more proximal arterial vessels in which resistance decreased by some 10 % A special test expt on the papaverine-relaxed vascular bed demonstrated that total and segmental vascular resistances if anything were somewhat smaller during pulsatile than non pulsatile perfusion This indicates that the resistance decline during non-pulsatile perfusion as depicted in Fig 3 was caused by active smooth muscle responses and not by passive effects for instance related to theological or other physical phenomena (cf Mellander & Arvidsson 1974)

The demonstrated clear inhibition of normal basal tone upon elimination of the arterial pulsations at maintained normal mean distending pressure thus seems to be an effect mainly localized to the microcirculation Such oscillations thus appear to be essential for the development of normal microvascular basal tone and the effect might result from repetitive positive dynamic transmural pressure stimuli on the vascular smooth muscle evoked during the rising phase of the pulse pressure curve

Effects of arterial pressure reduction on basal vascular tone and possible underlying mechanisms Observations of total and segmental vascular resistances in the adrenergically blocked muscle region were performed on 5 cats during graded reductions of arterial inflow pressure which was accomplished by adjustments of a screw-clamp placed around the arterial shunt tubing (see Methods) proximal to the site of the arterial pressure measurement Such compression of the tubing produced not only a decrease of mean arterial blood pressure from its control value of about 140 mmHg in these expts but also a simultaneous gradual attenuation of the arterial pulse pressure oscillations which were virtually abolished at a mean pressure of 40 mmHg Mean arterial pressure was reduced to the various levels depicted on the abscissa in Fig 4 and this was done for short periods of time (<60 s) to minimize possible metabolic vasodilator influence The resistance data on the ordinate in Fig 4 refer to the steady state after such a short-lasting pressure reduction Complete recovery of vascular tone was instituted between successive pressure reductions

It can be seen from the figure that total vascular resistance in the muscle region (R_{tot}) decreased with decreasing arterial inflow pressure This resistance decline was steepest in the pressure range down to about 80 mmHg and implied a fairly well developed autoregulation of blood flow in this pressure range Note that the decline in total vascular resistance could be almost entirely explained by dilation in the microvessels the basal tone of which was almost completely abolished (inhibited by almost 80% of maximum) at the arterial pressure of 40 mmHg Resistance in the proximal arterial vessel and the large veins remained fairly constant or at the low arterial pressure levels even tended to increase with decreasing arterial pressure The latter effect might mainly be ascribed to passive elastic recoil in these quite distensible vessels (cf Caro et al 1978) during the reduced arterial pressure which thus possibly could have obscured some minor concomitant inhibition of their basal tone

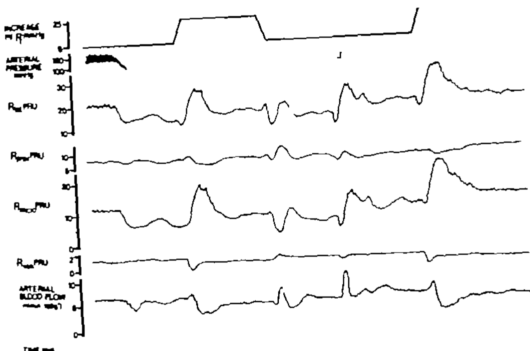


Fig. 5. Experiment serving to illustrate that basal tone in the microvessels (R_{micro}), which is markedly decreased by reduction of arterial pressure, can be partially restituted by experimental restoration of mean distending pressure to normal accomplished by superimposed increase of transmural pressure (P_t) = decrease of extravascular pressure. The experiment also demonstrates that dynamic and static myogenic reactivity is quite well preserved during the induced arterial hypotension. For further details see text.

It is concluded that basal vascular tone in the microvessels is distinctly dependent upon the arterial pressure level and an attempt will be made to elucidate the underlying mechanisms. It can be deduced from the data in Fig. 3 that a significant part of the described inhibitory effect on microvascular tone of arterial pressure reduction can be attributed to the concomitant experimental attenuation of the arterial pulsations. Some 35% of the inhibitory effect could be ascribed to the latter factor at the lowest arterial pressure level (40 mmHg), where the pulsations were virtually eliminated. The remaining inhibitory effect might be attributed to several factors, for instance to decreased static transmural pressure distension caused by fall of mean arterial pressure, to decreased dynamic transmural pressure stimuli (see Discussion), to possible metabolic inhibitory influence on basal tone and/or on myogenic reactivity etc.

The expt depicted in Fig. 5 provides some information about the importance of static transmural pressure distension for vascular tone and about static and dynamic myogenic reactivity during arterial pressure reduction. In this expt. arterial pressure was reduced from about 150 to 100 mmHg, i.e. in the range where blood flow was reasonably well autoregulated to minimize possible metabolic dilator influence. Flow (mean value) was thus reduced by only some 12% in this case. Total vascular resistance (R_{tot}) decreased by about 5.5 PRU during the pressure reduction, almost entirely explained by inhibition of basal tone in the microvessels.

This arterial pressure reduction led to a fall of mean distending pressure in the whole vascular bed by about 25 mmHg, calculated as (AP+VP)/2 and to attenuation of the pulse pressure oscillations. The low mean distending pressure could now, under maintained arterial hypotension, be roughly restored to normal by increasing overall vascular transmural pressure = decrease of plethysmographic (extravascular) pressure by the same amount, a procedure shown in Fig. 5. This compensatory increase of transmural pressure was applied at the rate of $\sim$5 mmHg/s and led to an initial pronounced dynamic myogenic constrictor response in the microvessels which raised vascular

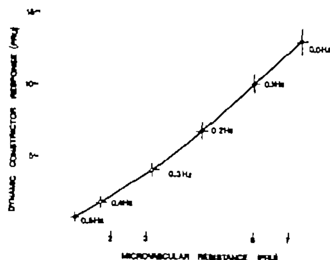


Fig 6 Effects of simulated light muscle exercise (low frequency somatomotor fibre stimulation) on microvascular tone (abscissa) and on the superimposed dynamic myogenic constrictor response (ordinate) to a standardized test transmural pressure stimulus (pressure increase by 40 mmHg applied at the rate of 2.5 mmHg/s) Note the parallel decrease in microvascular tone and in the dynamic constrictor response with increasing rates of somatomotor fibre excitation

tone also in the whole vascular bed above the control level at normal arterial pressure The evoked dynamic constrictor response was of almost the same magnitude as that observed at normal arterial pressure (see latter part of Fig. 5) demonstrating that dynamic myogenic microvascular reactivity was quite well preserved during the arterial pressure reduction (cf Grände 1979)

When static mean distending pressure was restored i e in the period of constant increased vascular transmural pressure (Fig 5) small though clearcut static myogenic constrictions developed in the microvessels as well as in the proximal arterial vessels which raised tone in the whole vascular bed by about 1 8 PRU Such experimental restoration of static vascular transmural pressure distension (but not of the pulse pressure oscillations) during lowered arterial pressure thus led to a partial but not complete recovery of basal vascular tone The average of 5 such expts indicated that basal tone in this way could be restored to almost 35% in the whole vascular bed and to about 35% in the microvessels of what was lost during the initial arterial pressure reduction The static myogenic response during lowered arterial pressure was of almost the same order of magnitude as during normal arterial pressure (see latter part of Fig 5) indicating that static *myogenic* reactivity was quite well preserved during the arterial hypotension

Return of plethysmographic pressure to zero (Fig. 5) caused an initial marked dynamic microvascular dilator response after which tone returned to the previous low level instituted by the arterial pressure decline Release of arterial pressure to the control level finally evoked an initial quite pronounced dynamic microvascular constriction after which tone via an oscillatory behaviour returned to the control level in all sections of the vascular bed

In some additional expts similar to that depicted in Fig. 5 overall vascular transmural pressure was raised from an AP level of about 80 mmHg by 65 mmHg corresponding to the normal mean distending pressure at an AP of 125 mmHg. This resulted in an average static constrictor response in the microvessels of about 2 8 PRU and in the proximal arterial vessels of about 0 8 PRU The latter data might serve as rough estimates of the extent to which the static distension effect of normal mean arterial pressure contributes to the establishment of normal basal vascular tone

These data and those presented in Figs ? and 5 provide strong circumstantial evidence for the opinion that basal vascular tone especially in the microvessels during normal arterial blood pressure to a significant extent is dependent both on its static distension effect related to mean arterial pressure and on its pulse pressure oscillations Yet these two factors do not seem to be the only ones responsible for the initiation of basal vascular tone (see Discussion)

The decline in microvascular tone during arterial pressure reduction as illustrated in Fig 5 might in part be explained by the action of metabolic dilator factors accumulating during the resulting flow reduction even if it was small in this case The fact that static and especially dynamic myogenic reactivity was shown to be quite well preserved during the arterial pressure reduction suggests that such an influence was small unless the metabolic control system interferes with basal tone via some mechanism completely unrelated to myogenic reactivity The latter po sibility was examined in a special series of expts by determining myogenic reactivity in terms of its prominent dynamic component under more defined activation of the metabolic control sy tem of keletal

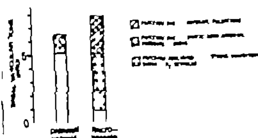


Fig. 7 Histogram summarizing in approximate figures the extent of normal basal tone in proximal arterial vessels (>25 μm d) and microvessels (<25 μm) in skeletal muscle (total height of columns) and its dependence on transmural pressure stimuli (see key). Note that considerable fraction of the normal basal tone in the microvessels dependent on arterial pressure, both via its static distension effect related to mean arterial pressure and its dynamic pulse pressure distension. Note further that a strong inhibitory dynamic transmural pressure (P) stimulus (dP/dt 5 mmHg/s) could virtually abolish basal tone in these vessels. Basal tone in the proximal arterial vessels was much less affected by such transmural pressure stimuli.

muscle, viz. by graded somato-motor fibre excitation.

Fig. 6 summarizes the results of 7 expts in which observations of microvascular tone and of the dynamic myogenic constrictor response to a standardized transmural pressure increase at a rate of 2.5 mmHg/s were performed under simultaneous somato-motor fibre stimulation in the low frequency range up to 0.5 Hz, simulating light exercise (cf. Lundvall 1972). It can be seen that such metabolic influence in fact decreased microvascular tone and the dynamic myogenic constrictor response in parallel, and that these effects were graded in relation to the excitation rate with close to maximal inhibitory effects evoked at 0.5 Hz.

It is concluded that the metabolic control system distinctly interacts with the mechanism(s) responsible for dynamic myogenic reactivity at least when activated by skeletal muscle contraction. It therefore appears that metabolic influence did not significantly contribute to the inhibition of basal microvascular tone during the moderate arterial pressure reduction produced in Fig. 5 since myogenic reactivity was then well preserved (cf Grande 1979). This does not refute the possibility that metabolic influence can be of significant importance during more pronounced and longlasting arterial hypotension.

Mention should finally be made that the proximal arterial vessels were shown to be much less sensitive than the microvessels to the metabolic influence of muscle contraction (cf Folkow et al. 1971), basal tone in the former vessels being virtually unaffected by somato-motor fibre stimulation in the range up to 0.5 Hz. Stronger metabolic stimuli, however caused gradual inhibition of this tone also maximal relaxation in these vessels being observed at excitation rates around 4 Hz, corresponding to the metabolic influence of heavy exercise (cf Lundvall 1972).

DISCUSSION

The aim of the present study was to elucidate in some detail the characteristics of the intrinsic basal vascular tone in skeletal muscle with regard to its extent and site along the vascular bed its dependence on static and dynamic transmural pressure stimuli and its sensitivity to local metabolic influence. A method was used which according to previous evaluation (see Grande et al 1977 Grande 1979) seems to provide quite reliable determinations of total vascular resistance as well as segmental resistances in what is denoted proximal arterial vessels microvessels and large veins The data indicated that the site for separation between proximal arterial vessels and microvessels referred to an approximate vessel diameter as small as 25 μm implying that the former vessels extended somewhat into the domain of the microcirculation. The described active resistance reactions in the microvessels of this study can be considered to refer to the terminal arterial vessels mainly even if the capillaries and minute venous vessels were also included in these resistance measurements (Grande et al. 1977).

The results first confirmed previous observations (see Mellander & Johansson 1968) that basal tone in the adrenergically blocked vascular bed under control conditions was high on the arterial side and low in the large veins as evidenced by comparison of segmental vascular resistances at normal tone and after complete smooth muscle relaxation with papaverine The main results of the present study therefore deal with the characteristics of basal tone in the proximal arterial vessels and the microvessels and they may be summarized schematically and in rough figures, as illustrated in Fig. 7 The extent of smooth muscle tone is here expressed in

terms of its active vascular resistance corollary (PRU) i e the resistance at the normal level of basal tone minus the small physical' resistance remaining after complete smooth muscle relaxation

The following main conclusions may be drawn Under control conditions basal tone corresponded to an average of 6 6 PRU in the proximal arterial vessels and 7 8 PRU in the microvessels The normal arterial blood pressure seemed to be partially responsible for the initiation and maintenance of this basal tone both via its pulse pressure oscillations and its mean pressure level the former quite likely creating a repetitive dynamic and the latter a static transmural pressure stimulation of the smooth muscle Note however that the fraction of basal tone which could be attributed to these two stimuli was much greater in the microvessels than in the proximal arterial vessels (Fig 7) Experimental application of a strong (probably maximal) inhibitory dynamic transmural pressure stimulus ($dP_T/dt = -5$ mmHg/s) caused a very effective though not complete inhibition of the normal basal tone in the microvessels but such a stimulus had only a slight effect on tone in the more proximal vessels (Fig 7)

Mention should be made in this context that dynamic transmural pressure stimulation of the vascular bed might not solely be affected by pulse pressure oscillations but perhaps also by segmental pressure oscillations in the longitudinal direction created for instance by the intermittent local constrictions and dilations denoted vasomotion

The demonstration that normal microvascular basal tone to such a significant extent is depending upon static and dynamic transmural pressure stimuli created by the normal arterial blood pressure might offer a very simple explanation of myogenic autoregulatory phenomena in the microcirculation Fall of arterial blood pressure within a certain range would lead to a graded inhibition of basal tone simply due to partial elimination of its initiating static and dynamic pressure stimuli Conversely raised arterial pressure might lead to reinforced vascular tone due to increased static mean pressure distension and to the usually associated increase in the amplitude and/or frequency of the pulse pressure oscillations or other possible mechanisms facilitating the dynamic transmural pressure stimuli The fact that basal tone in the proximal arterial vessels was largely independent of arterial blood pressure per se might then possibly explain why autoregulation of flow occurs only within a limited range of arterial pressures.

The described results which demonstrate that basal tone is much more dependent upon and sensitive to transmural pressure stimuli in the microvessels than in the proximal arterial vessels suggest that basal tone is of somewhat different nature in these two sections The site for such a possible functional differentiation of the smooth muscle along the arterial vascular tree is probably not precisely at the vessel dimension of 25 μm (i.d.) but perhaps at a more proximal level the small fraction of basal tone sensitive to transmural pressure stimuli in what was here called proximal arterial vessels (Fig 7) thus might reflect the behaviour in their smallest ramifications which should be denoted microvessels rather than proximal vessels Whatever the case the fact remains that the by far largest fraction of basal tone in the microvessels (< about 25 μm i d) was sensitive to transmural pressure stimuli while the by far largest fraction of basal tone in the more proximal vessels seemed to be irresponsive to such stimuli. Another difference between the proximal arterial vessels and the microvessels was that basal tone in the latter (Fig. 6) was much more sensitive to metabolic stimuli than that in the former (cf Folkow et al 1971) Even if basal tone in both sections might be considered to be of myogenic origin (see Introduction) the described functional discrepancies might suggest somewhat different cellular mechanisms behind the basal tone in the proximal and distal arterial vessels a possibility which will be discussed in the light of some recent electrophysiological studies on vascular smooth muscle

The cellular mechanisms responsible for basal vascular tone and myogenic reactions are not yet experimentally defined in the arterial microvessels specifically but a recent in vitro study (Johansson & Mellander 1975) has clarified the electrophysiological events behind myogenic reactivity to mechanical stimuli in the single unit' spike generating vascular smooth muscle of the rat portal vein which seems to have several characteristics in common with arterial microvascular' smooth muscle The myogenic contractile response pattern is thus quite similar in both these types of vascular smooth muscle (Johansson & Mellander 1975 Grände et al 1977 1978) Both exhibit a static as well as a dynamic component in their myogenic responses of which the latter component is the

much more prominent one a maximal excitatory dynamic stimulus was thus some 15 times more effective in causing contraction in vitro and microvascular constriction in vivo than a maximal static stimulus. A strong inhibitory dynamic stimulus was furthermore capable to abolish contractile activity in the portal vein and, as shown in this study also could effectively inhibit basal microvascular tone in vivo. Such close resemblance between the portal vein and the arterial microvessels with regard to their myogenic contractile patterns of response and stimulus-effector characteristics makes it quite likely that the myogenic responses in both types of muscle are mediated via some common cellular mechanism.

The portal vein shows a characteristic spontaneous regular rhythmic contractile activity in the control state at low preload which is caused by bursts of action potentials primarily generated from a pacemaker cell (Johansson & Mellander 1975). When it was exposed to excitatory static or dynamic stimuli (passive stretch at graded rates) spike discharge and myogenic mechanical activity increased in parallel The dynamic electrical and mechanical responses were graded in relation to the rate of length (L) change, dL/dt Similarly there are graded decreases in electrical and mechanical activity in response to the rate of passive shortening (negative dL/dt) A strong inhibitory dynamic stimulus caused complete abolition of electrical and mechanical activity a finding which deserves special attention in the interpretation below Excitatory and inhibitory myogenic effector reactions in this spike-generating type of vascular smooth muscle are thus mediated via facilitation and inhibition of normal pacemaker generated spontaneous spike discharge

It follows from the considerations above that the myogenic reactions to transmural pressure stimuli in the arterial microvessels quite likely might be mediated via variations in pacemaker spike discharge as well (even if the size of their single units must be much smaller than in the portal vein). If this conclusion is valid it would imply that at least a major fraction of the basal tone in the microvessels is dependent upon action potentials, viz. that fraction (see Fig. 7) which was abolished by the inhibitory dynamic transmural pressure stimulus (dP_T/dt −5 mmHg/s). Part of the normal basal tone in these vessels might then be due to a spontaneous pacemaker discharge (like in the portal vein) and parts initiated by the normal arterial pressure via its static and dynamic transmural pressure stimuli (Fig. 7), leading to continuous reinforcement of electrical activity in the control situation.

Basal tone in the proximal arterial vessels was little affected by transmural pressure stimuli (Fig. 7) and its major fraction might therefore be virtually independent of action potentials. Smooth muscle in these vessels might then be mainly of the same non-spike-generating type as demonstrated in large arterial vessels in vitro (Su Bevan & Ursillo 1964 Hermsmeyer 1971 Somlyo Vinall & Somlyo 1971 von Loh & Bohr 1973 Casteels et al. 1977 Johansson 1978) where tone is considered to be caused by contracture and established by graded membrane depolarization. The small fraction of basal tone in the microvessels which remained upon exposure to the strong inhibitory transmural pressure stimulus (see Fig. 7) might possibly be explained by a similar mechanism. The suggested differentiation of smooth muscle along the arterial vascular bed in skeletal muscle might therefore not be very distinct characteristic (cf in vitro studies by Roddie 1960, Steedman 1966, Biamino & Kruckenberg 1969) but include an element where the difference is mainly of quantitative nature Intrinsic basal tone in the large veins was low and normal resting tone might here be established by action potentials initiated by extrinsic especially adrenergic stimuli to their smooth muscles which seem to be more of the multi-unit type (cf Mellander & Johansson 1968)

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Efflux of cyclic AMP prostaglandin E_2 and $F_{2\alpha}$ and thromboxane B_2 in leg lymph of rabbits after scalding injury

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JONSSON C-E, SHIMIZU Y FREDHOLM B B GRANSTRÖM, E. & OLIW E. Efflux of cyclic AMP prostaglandin E_2 and $F_{2\alpha}$ and thromboxane B_2 in leg lymph of rabbits after scalding injury Acta Physiol Scand 1979 107 377–384. Received 2 July 1979 ISSN 0001-6772. Department of Plastic and Reconstructive Surgery and Surgical Research Laboratory Karolinska sjukhuset, Stockholm, and Departments of Pharmacology and Chemistry Karolinska institutet, Stockholm, S eden.

Leg lymph was collected from pentobarbital anaesthetized rabbits before and after scalding injury of the paw (75°C for 20), and the contents of cyclic AMP (cAMP), prostaglandin E_2 (PGE_2) and $PGF_{2\alpha}$ and thromboxane B_2 (TXB_2) in lymph were determined. After injury lymph flow increased about four times. The maximal rate of flow was found between 30 and 60 min after scalding The efflux of cAMP and immunoreactive $iPGE_2$, $iPGF_{2\alpha}$ and TXB_2 also increased. The maximum values were detected at approximately 0–30, 30–60, 30–60 and 180–240 min, respectively after the injury The output of cAMP $iPGE_2$ and $iPGF_{2\alpha}$ and TXB_2 in lymph of the contralateral non-scalded paw remained low throughout the experiments. When rabbits were injected with indomethacin (2.5 mg/kg) or diclofenac sodium (2.5 mg/kg) immediately after the scalding injury the efflux of cAMP $iPGE_2$ and $iPGF_{2\alpha}$ were low Lymph flow was markedly reduced after treatment with diclofenac sodium, treatment with indomethacin did not significantly affect lymph flow The results suggest prostaglandin-dependent formation of cAMP following scalding injury which may be related to the initial responses to scalding.

Key words Scalding injury lymph flow cAMP thromboxanes, prostaglandins

Scalding injury is followed by vascular reactions, ie. erythema and edema formation, the extent of which depends on the duration and magnitude of the increased temperature (Arturson 1961) In the acute phase of burn there is a rapid loss of intravascular fluid to the injured tissue mainly due to vasodilatation, increased microvascular permeability and increased extravascular osmotic pressure (Arturson & Mellander 1964) Once edema is formed blood flow seems to be reduced in the injured tissue (Hamar et al. 1979) For a long time it has been assumed that the effects of scalding are mediated by vasoactive compounds formed or released to the tissues. For example, there is evidence to suggest that histamine (Edery & Lewis 1963) and prostaglandins mainly prostaglandin E_2 (PGE_2) (Willis 1970, Jonsson 1971 Williams 1979) mediate the initial vascular response

Recently intermediates in the biosynthesis of prostaglandins, the prostaglandin endoperoxides (PGG_2 and PGH_2) were isolated (Hamberg & Samuelsson 1973 Nugteren & Hazelhof 1973). The availability of the prostaglandin endoperoxides led to the discovery of two unstable and very potent products, thromboxane A_2 (TXA_2, Hamberg et al.

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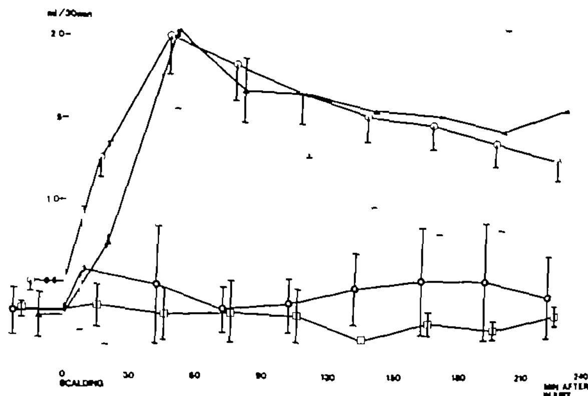


Fig 1 Leg lymph flow before and after scalding injury At 0-time the paw was injured by immersion in water of 75°C for 20 s) Lymph was collected in 30 min periods in every experiment However for purposes of clarity of presentation the points in the graph have not all been plotted at the mid point of each collection interval Non-treated rabbits $n=12$ O—O bars denote S E. Lymph from non-scalded leg □—□ $n=$ mean value and range Rabbits treated immediately after scalding with indomethacin (2.5 mg/kg, $n=2$, ▲—▲) or diclofenac sodium (2.5 mg/kg, $n=2$ **O—O** mean values and ranges are given)

1975) and prostacyclin PGI (Moncada et al 1976) Although the prostaglandin endoperoxides are precursors of prostaglandins as well as of the thromboxanes and PGI_2 the conversion of the prostaglandin endoperoxides to their different products varies between tissues (Pace-Asciak 1977 Vane 1978) For example vessel walls and heart may convert the endoperoxides mainly to PGI (Moncada & Vane 1979) while platelets and lung tissue convert them mainly to TXA_2 (Hamberg et al 1977) In other tissues "primary prostaglandins PGD_2 PGE_2 and $PGF_{2\alpha}$ are the dominating products (e g Fredholm & Hamberg 1976 Oliw et al 1979) The different products of endoperoxide metabolism display a wide variety of biological effects. Thromboxane is for example a potent vasoconstrictor PGI_2 is a potent vasodilator and of the "primary prostaglandins, PGE_2 is a vasodilator while $PGF_{2\alpha}$ may cause venoconstriction (Svensson & Fredholm 1977 Samuelsson et al 1978*a*) There is considerable evidence that cyclic AMP (cAMP) mediates several effects of prostaglandins (Samuelsson et al 1978*b*) In particular it is an interesting possibility that vasodilatation induced by PGI_2 and PGE_2 is due to stimulation of cyclic AMP production in vascular smooth muscle since cyclic AMP is known to mediate vasodilatation due to several agents (see Bar 1974 for review)

In this communication we report studies on the efflux of cAMP prostaglandins and thromboxanes in leg lymph of rabbit after scalding injury of the paw Thromboxane B (TXB_2) a degradation product of the very unstable thromboxane A (TXA_2) was determined as an indicator of TXA_2 synthesis

MATERIALS AND METHODS

Rabbits weighing between 2 0 and 5 0 kg were anaesthetized with sodium pentobarbital (18 mg/kg). Additional

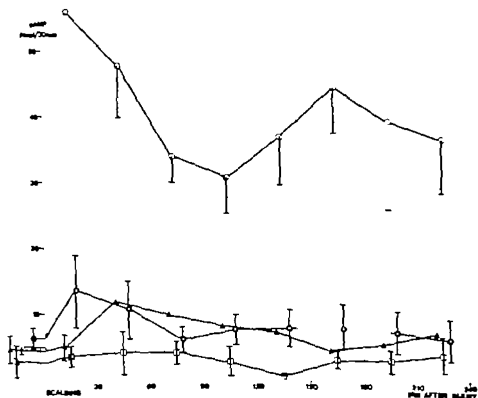


Fig. 2 cAMP in lymph before and after scalding injury expressed as pmol per 30 min periods in every experiment. However, for purposes of clarity of presentation the points in the graph have not all been plotted at the mid point of each collection interval. Lymph from non-treated rabbits, n=5 O—O mean and S.E. given. Lymph from control leg, n=2 □—□. Rabbits treated with indomethacin (n=2 ▲—▲) or diclofenac sodium (n=2 ●—●). Mean values and ranges are given in these experiments.

doses were given when required. For visualization of the lymph vessels, Fluorescein Dextran (FITC-Dextran 150, Pharmacia, Sweden) 25 mg in 0.5 ml saline, was injected into the subcutaneous tissue of the paw. A leg lymph vessel was cannulated as described (Jonsson & Shanler 1979). Lymph flow was facilitated by gentle massage or squeezing of the paw every 5 s. Blood from the carotid artery was used for assay of cAMP in plasma. Ice-chilled centrifuge tubes (containing EDTA) were filled with blood (2 ml). After centrifugation plasma was pipetted off and frozen.

Lymph was collected in 30 min periods in ice-chilled glass tubes (1 ml). For assay of cAMP the tubes contained 50 µl 0.5 M EDTA (final concentration about 25 mM). The tubes were kept at −20°C before assay which was usually performed within two weeks. Lymph volume was estimated by weighing the tubes before and after collection of lymph. The scalding injury was effectuated by immersion of the paw in water of 75°C for 20 s.

Indomethacin (2.5 mg/kg, Merck Sharp & Dohme, Rahway USA) and diclofenac sodium (2.5 mg/kg, o-(2,6-dichlorophenyl-amino)-phenylacetate sodium, Ciba-Geigy Basel, Switzerland), were freshly prepared in 0.1 M potassium phosphate buffer pH 8.0, immediately before use. The drugs were injected intravenously immediately after the scalding injury.

Cyclic AMP. Assays of cAMP were conducted in duplicate according to the method of Brown et al. (1972) using (8-^{3}H)-cyclic AMP (26 Ci/mmol, The Radiochemical Centre, Amersham). Binding protein was prepared from bovine adrenal cortex, and Norit G SX (N V Algemeene Norit, Amsterdam) was used to separate free and bound cyclic nucleotide. In order to validate the assay some samples were assayed also by radioimmunoassay (using reagent kit from New England Nuclear Boston), after dilution and acetylation as described by Harper & Brooker (1975).

Results were virtually identical in samples of intact lymph and in lymph deproteinized with trichloroacetic acid followed by 5 times extraction with ether (13.6±0.4 pmol/ml vs. 14.8±1.4 pmol/ml, n=10). The lymph bound less than 1.5% of the cAMP in the absence of exogenous binding protein. Deproteinization was therefore not routinely carried out. Similarly purification of cAMP on

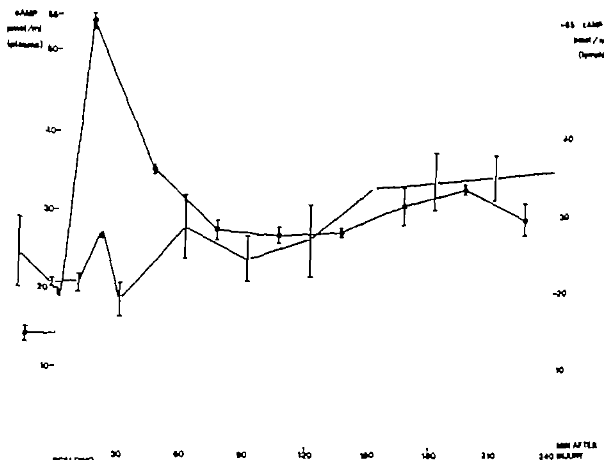


Fig 3 cAMP (pmol/ml) in lymph (●—●) and plasma (O—O) before and after scalding Mean values (n=) and range (bars) are given

Dowex 50 columns did not alter the values. Finally treatment with *phosphodiesterase* removed 90 % or more of the binding activity

On the other hand it was found to be necessary to treat the lymph samples immediately with EDTA (5–20 mM) in order to preserve the cAMP Samples obtained and stored in the absence of EDTA contained substantially less cAMP (3 7±0 6 pmol/ml vs 15 4±1 4 pmol/ml) In order to further study the breakdown of cAMP ³²P cyclic AMP (2 pmol/ml) (New England Nuclear Boston 32 7 Ci/mmol) was incubated with lymph in the presence or in the absence of EDTA for varying lengths of time at 0°C 20°C or 37°C In the presence of EDTA (5 mM) less than % of the cAMP was hydrolyzed in 20 min In the absence of EDTA 8–10 % was broken down in 20 min at 20°C or 37°C These findings indicate that less than 10 % of the cAMP content of lymph is likely to be broken down during collection of samples and that provided that EDTA is present, very little breakdown will occur during further processing of the samples.

Prostaglandins and thromboxanes PGE_2, $PGF_{2\alpha}$ and TXB were measured in duplicate in unextracted lymph by radioimmunoassay $PGF_{2\alpha}$ was measured using a $PGF_{2\alpha}$-antiserum while PGE_2 was reduced to $PGF_{2\alpha}$ and $PGF_{2\beta}$ with NaBH and measured using an antiserum raised against $PGF_{2\beta}$ (Oliw et al. 1978 Oliw 1979). TXB_2 was determined by radioimmunoassay as previously described (Granström et al 1976) Due to the very low amount of lymph no conclusive identification of the compounds could be performed and the values obtained by radioimmunoassay are referred to as $iPGE_2$, $iPGF_{2\alpha}$ and $iTXB_2$ i e immunoreactive PGE_2, $PGF_{2\alpha}$ and TXB_2, respectively in the text

RESULTS

Lymph flow Before scalding injury lymph flow was approximately 0 5 ml/30 min and increased to a peak of about 2 0 ml/30 min 30–60 min after the injury (Fig 1) The lymph flow decreased somewhat after 1 h However at 4 h after the injury the flow was still above control values Lymph flow in the control leg did not show any significant change during the experiments

Cyclic AMP The rate of cyclic AMP appearance in lymph was initially low (approximately 5 pmol/30 min) but increased ten fold immediately following scalding and stayed elevated for the rest of the experiment (Fig)

Before scalding injury the lymph cyclic AMP

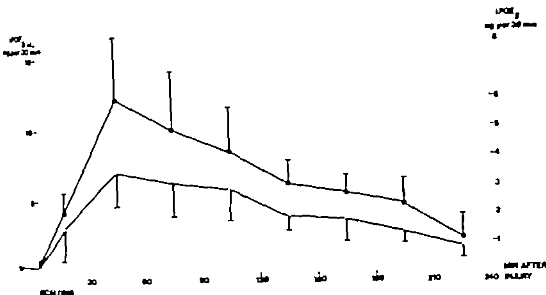


Fig 4 Efflux of iPGE$_2$ (O—O) and iPGF$_{2\alpha}$ (●—●) in lymph before and after scalding. Mean values (=5) and S.E. (bars) are given.

levels were lower than those in plasma (14 1±4.5 pmol/ml (mean, range, =2) vs. 25.6±4.4 pmol/ml, =4). Already in the first sample after scalding, the lymph cyclic AMP level was increased more than three-fold, whereas the plasma cyclic AMP concentration was essentially unchanged. Thereafter the lymph cyclic AMP concentration decreased progressively and after one hour the lymph levels were close to those found in plasma, which instead progressively increased after the scalding injury (Fig. 3).

Prostaglandins The levels of PGE$_2$ and iPGF$_{2\alpha}$ were below the detection limit before scalding injury (Fig. 4). After the injury both iPGE and iPGF$_{2\alpha}$ were detected. Maximum levels were found 30–60 min after scalding and corresponded to an efflux of 8 and 12 ng/30 min into lymph for iPGE$_2$ and iPGF$_{2\alpha}$ respectively After that time the rate decreased, but 4 h after the injury immunoreactive material was still detectable.

Thromboxane Before and 0–30 min after scalding the output of TXB$_2$ in lymph was low (0 4 ng/30

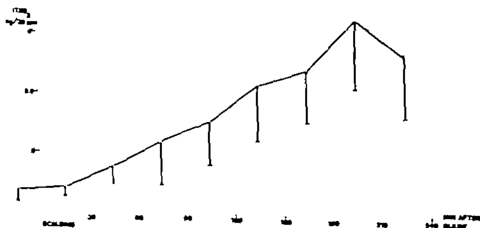


Fig 5 Efflux of iTXB$_2$ in lymph before and after scalding. Mean values (*n* 4) and S.E. (bars) are given.

min) (Fig 5) The rate gradually increased and the highest levels were found 180–240 min after the injury or even later in a few animals

Effect of indomethacin and diclofenac sodium After treatment with diclofenac sodium (2 5 mg/kg $n=2$) directly after the scalding injury the lymph flow was very low (Fig 1) On the other hand in two rabbits treated with indomethacin (? 5 mg/kg) lymph flow still increased after the injury In an earlier study in dogs indomethacin caused a marked suppression of lymph flow (Arturson & Jonsson 1973) The failure to observe a significant effect in the present experiments may have been fortuitous and caused by the small number of experiments in which paired controls were unobtainable The rate of cAMP release into lymph was markedly reduced by both drugs (Fig 2) However even after pre treatment with those drugs a significant two-fold increase in cyclic AMP overflow in lymph was detected $iPGE_2$ and $iPGF_{2\alpha}$ were below levels of detection after treatment with either drug

DISCUSSION

The results demonstrate increased efflux of cAMP $iPGE_2$ $iPGF_{2\alpha}$ and $iTXB_2$ in leg lymph after scalding injury of the paw The compounds appeared in lymph in a characteristic sequence Maximum secretion of cAMP appeared during the first 30 min period followed by $iPGE_2$ $iPGF_{2\alpha}$ (maximum 30–60 min) and $iTXB_2$ (maximum 180–240 min after injury or later) Lymph flow also increased and reached its level between 30 and 60 min following injury

The very small levels of the compounds found in control lymph (Fig 2) indicate that the compounds were formed in the injured tissue and were not filtered out from the blood circulation As for cAMP this assumption was strengthened by the finding that lymph levels of cAMP increased above the plasma levels On the other hand the late increase in lymph cAMP co-incided with a rise in the plasma level (Fig 3) and may have been caused by filtration from plasma. The increased efflux of $iPGE_2$ and $iPGF_{2\alpha}$ reflects an increased prostaglandin biosynthesis in the injured tissues (Hamberg & Jonsson 1973) The efflux of TXB_2 indicates that the same is most probably true for TXA_2 as capacity for TXA_2 biosynthesis has been described in many tissues (Samuelsson et al. 1978*b*)

Before discussing the significance of these results it must be pointed out that the values are based on radioimmunoassay Because of the small amounts of lymph recovered conclusive identification of the compounds could not be carried out. Indomethacin and diclofenac sodium inhibit biosynthesis of prostaglandins in vitro (Vane 1971 Ku et al 1975) as well as in vivo in the rabbit (Oliw et al 1978) When the rabbits were treated with either of these drugs the efflux of $iPGE_2$ and $iPGF_{2\alpha}$ in lymph was markedly reduced supporting the assumption that the measured immunoreactive activity in lymph was in fact related to prostaglandin endoperoxide synthesis

The initial vascular response to acute injury is vasodilatation (Arturson & Mellander 1964) Vasodilatation induced by several different agents may be caused by increased levels of smooth muscle cyclic AMP (Bär 1974) The markedly increased production of cAMP may thus reflect the effect of vasodilatory compounds on the arterial smooth muscle although other sources than smooth muscle are likely to contribute to lymph cAMP

There is evidence that the vasodilatation is abolished or considerably reduced by anti-inflammatory drugs (Spector & Willoughby 1959 Arturson & Jonsson 1973) It is therefore interesting that two structurally unrelated anti inflammatory drugs considerably reduced the efflux of cyclic AMP into lymph As these drugs inhibit prostaglandin biosynthesis it is tempting to speculate that the vasodilatation and cyclic AMP overflow is prostaglandin mediated However a slight but significant elevation of cyclic AMP overflow was seen also in the presence of the PG-synthesis inhibition suggesting that also other material capable of stimulating cyclic AMP formation is formed following scalding. Since there was no increase in cyclic AMP efflux in the contralateral paw this PG-unrelated compound could not be a circulating agent or be released following a generalized nerve activation The nature of this unknown local factor remains unknown

However the fact remains that most of the cyclic AMP production of the scalded paw could be abolished by PG-synthesis inhibitors

The most pronounced cyclic AMP overflow was found during the first 30 min after scalding. On the other hand $iPGE_2$ appeared later It is therefore possible that some other product of prostaglandin-endoperoxides was responsible for the cyclic AMP

increase (and the vasodilatation). It is known that PGI_2 is a potent vasodilator (Moncada & Vane 1979). It is also known that this unstable product is a potent stimulator of cyclic AMP production in several cell types (Gorman et al. 1977 Tateson et al. 1977 Hjemdahl et al. 1978). It is an interesting possibility that PGI_2 may be responsible for some neural effects of scalding. We are not aware of any direct evidence that PGI is produced by scalded skin. However an unidentified peak of smooth muscle stimulating material was detected by Hamberg & Jonsson (1973). This material was more polar than PGE and $PGF_{2\alpha}$ and may have been identical with 6-keto-$PGF_{1\alpha}$ the stable breakdown product of PGI. For the above reasons and because of the potent hyperalgesic affect of PGI (Higgs et al 1978) attempts to demonstrate PGI following scalding are definitely called for

Vasodilatation in the injured tissue is followed by a state of decreased blood perfusion (Hamar et al. 1979). The simplest explanation for this may be compression of the vessels by the edema. Since TXA_2 has very potent vasoconstricting effects, and the decrease in lymph flow co-incided with increasing levels of TXB_2, it is tempting to suggest that TXA_2 may contribute to the vasoconstriction in the edema phase. The vascular effects of TXA_2 have not been studied in vessels in the paw and thus much work must be done before definitive conclusions can be drawn. It is interesting to note however that a role for TXA_2 in the production of inflammatory edema has been proposed (Kuehl et al. 1977 Chang et al. 1978)

In conclusion the present results demonstrate a marked increase in cyclic AMP efflux into lymph from the scalded rabbit paw This increase is due to local formation of the cyclic nucleotide and precedes the increase in lymph flow The increase in cyclic AMP overflow was considerably blunted by two different inhibitors of PG-synthesis suggesting that some product(s) of the endoperoxide synthetase reaction was responsible. Immunoreactive PGE_2 and $PGF_{2\alpha}$ were released into lymph but occurred later than cyclic AMP Thromboxane B_2, an indicator of thromboxane A_2, occurred even later following scalding. The possibility exists that the initial increase in cyclic AMP may have been caused by PGI_2. At any rate the results show that different products derived from PG-endoperoxides may be formed and released by injured tissue in a specific sequence.

This study was supported by grants from The Swedish Medical Research Council (nos. 2211 and 2553), Magnus Bergvalls stiftelse and Karolinska Institutets fonder

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Erroneous estimates of intrarenal blood flow distribution in the dog with radiolabelled microspheres

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Since the introduction of the radioisotope labelled microspheres (Ms) for measurement of regional cortical blood flow by McNay & Abe in 1970, the method has been extensively used in dogs under many experimental conditions. However several observations may suggest a skimming of 15 μm Ms at the offspring of the afferent arterioles from the interlobular arteries, resulting in lower Ms concentration in blood feeding the deep glomeruli than that feeding more superficial glomeruli (Katz et al 1971 Marknd et al. 1978 Clausen et al 1977 1979) Such skimming would be expected to be more pronounced for 15 than for 10 μm Ms (Phibbs & Dong 1970, Øfjord Clausen & Aukland 1979) Failure of Ms to enter deep arterioles in proportion to blood flow due to pure steric restriction seems excluded in the diameter range (Chenitz et al 1976, Marknd et al. 1978). We therefore decided to compare the distribution of 10 and 15 μm Ms in the dog kidney

Mongrel dogs, weighing 14–20 kg, were anesthetized by Mebumal, 25 mg/kg b wt In 3 expts (5 kidneys), midline abdominal incision was made and venous renoatrial shunt was established by polyethylene tube of 10 mm inner diameter Cannulas were placed in the shunt close to the kidney for blood sampling and pressure measurement. In 2 expts (4 kidneys) the kidneys were approached retroperitoneally and catheter inserted into the left renal vein via the spermatic vein for sampling of venous blood Nylon snares were placed loosely around both renal pedicles Blood flow in the left renal artery (electromagnetic flow probe) and arterial blood pressure were recorded. A mixture of 10 5 (S D 1 2) μm and 14 9 (S.D 1.0) μm microspheres (hereafter referred to as 10 μm Ms and 15 μm Ms) labelled with ^{85}Sr and ^{141}Ce respectively sonicated to ensure dispersion, were infused into the left ventricle through catheter inserted via the left common carotid artery The infused doses gave about 0 5 spheres of each type per glomerulus Blood sampling at constant rate of 12 ml/min was made from the aorta near the renal arteries through catheter inserted via femoral artery beginning prior to and lasting for 60 after Ms infusion. Sampling of renal venous blood at rate of 1 or 4 ml/min started simultaneously and was maintained for 3–5 min. The nylon snares were then tightened simultaneously and the undrained kidneys were excised, weighed and frozen in CO_2-alcohol mixture. Dissection of renal tissue samples were made from a sagittal 5 mm thick frozen slice cut out by saw 5–10 sectors were divided in 3 cortical zones of equal thickness (C C$_2$ and C$_3$) whereas the medulla was divided in 2 outer medullary zones (OM OM$_2$) and inner medulla (IM). The tissue samples weighed between 0 05 and 0.2 g. Radioactivity in arterial and venous blood and in tissue samples was counted in 3-channel well scintillation gamma counter (Searle Analytic Inc. Mod. 1185) for 10 min. Average renal blood flow per gram tissue was calculated from local flow rates and the respective zonal volumes taken to be: C 29% C$_2$ 23% C$_3$ 18% OM 12%, OM$_2$ 8% and IM 10% of total kidney volume. The relationship between the amount of Ms flowing into the kidney and the amount escaping through the renal vein was calculated directly from the arterial and venous sampling rates and blood radioactivities.

Mean arterial blood pressure averaged 125 (105–135) mmHg whereas renal venous pressure ranged from 3 to 9 mmHg. The renal extraction of Ms measured in 5 of the 9 kidneys was practically complete: 99.3 (S D 0.5)% and 99 9 (S D 0.06)% for 10 and 15 μm Ms, respectively As shown in Table 1 15 μm Ms gave higher flow in C but lower flow in all deeper zones as compared to 10 μm Ms. Since calculated total RBF was 6.5 (S.D 3.5)% higher with 10 μm Ms the zonal Ms concentrations in each sector were normalized to those of C in each kidney This procedure which obviates possible errors due to different isotope counting efficiencies in blood and tissue or to different Ms concentrations in sampled arterial and renal arterial blood, confirmed the disparity of the two intrarenal Ms distribution patterns (Fig. 1). The present results demonstrate beyond doubt that under the experimental conditions used here 15 μm Ms are greatly

Table 1 *Average onal blood flow and onal percentage of calculated total renal blood flow (RBF) obtained in 9 dog kidneys by microspheres (Ms)*

C C_2 C_3 Outer middle and inner cortex OM OM_2 Outer and inner halves of outer medulla. IM Inner medulla

Flow	MS	C	C_2	C	OM	OM	IM	C +M	RBF
ml/min g	10 μm	5 22	4 15	2 07	0.59	0 17	0 03	0 96	93
	15 μm	5.37	3 65	1 70*	0 4	0 04	0 02*	0 71	.74
% of RBF	10 μm	51 7	32 6	12 7	.4	0 44	0.10	15 6	
	15 μm	56 9*	30 7	11 2*	1 0*	0 15	0 06*	12 4*	

$P<0.01$ $P<0.05$ paired *t*-test.

under represented in deep cortex relative to 10 μm Ms Since it is very unlikely that 10 μm Ms should be overrepresented in blood flowing into the deep afferent arterioles as compared to blood in the inter lobular arteries It would appear that blood flow in deep cortex is underestimated and superficial corti cal flow is overestimated with 15 μm Ms It is of interest that similar results have recently been obtained in experiments on rats (Bankir 1979 Casellas & Mimran 1978 Aperia—personal communication 1979)

The results clearly indicate transglomerular pas sage of some 10 μm Ms into the medulla (Table 1) This prompted microscopic investigations as yet preliminary suggesting a postglomerular location of 5 to 30% of the cortical 10 μm Ms as compared to only a few per cent of the 15 μm Ms Calculations indicate that postglomerular interzonal passage of 10 μm Ms could not possibly account for more than a fraction of the disparate Ms distributions in the renal cortex We must therefore conclude that a different degree of skimming of 10 and 15 μm Ms along the interlobular arteries (Fig 2) is the main cause of the difference in Ms distribution Thus it seems highly dubious that 15 μm Ms or 10 μm Ms reflect true glomerular blood flow distribution in the dog kidney In view of the large number of studies on intrarenal circulation made by the radiolabelled Ms technique the crucial question is Do microspheres of 10 or 15μm diameter give reasonable

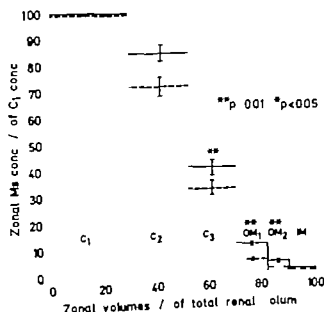


Fig 1 Relative concentrations of 15μm (broken lines) and 10 μm (solid lines) microspheres (Ms) in 3 cortical zones of equal thickness (C C C_3), in outer and inner halves of outer medulla (OM and OM_2) and in inner medulla (IM). Mean±S.D of 9 kidneys.

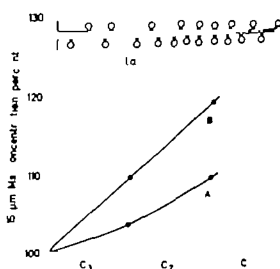


Fig 2 Estimated concentration of 15 μm Ms in interlobular arterial (i.l) blood reaching midcortex (C) and outer cortex (C) expressed in per cent of the initial i.a. concentration in inner cortex (C_3) The calculations are made on the assumption that true zonal glomerular flow is obtained by A 10 μm Ms or B ¹²⁵I-iodoantipyrine (Clausen et al 1979)

information on *changes* in blood flow distribution in the kidney? The results of Morkrid et al (1978) would suggest that vasodilation produced by lowering the renal perfusion pressure may reduce the skimming of 15 μm Ms more than that of 10 μm Ms. This is compatible with results from four preliminary experiments of the present design in dog kidneys during vasodilation by acetylcholine. In conclusion, until more is known about the distribution of Ms under various experimental conditions, it seems at least highly hazardous to estimate regional cortical blood flow with this indicator

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Immunohistochemical evidence for substance P immunoreactive nerve fibres in the taste buds of the cat

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Substance P was discovered by von Euler & Gaddum (1931) Forty years later Chang et al (1971) isolated and structurally characterized this factor as undecapeptide. Substance P was shown to have marked regional distribution both in the central nervous system and in peripheral tissues (Pernow 1953) and Lembeck (1953) suggested that it may be transmitter substance primary sensory neurons This view was strongly supported by the elegant biochemical and electrophysiological experiments of Otsuka and colleagues (see Otsuka & Takahashi 1977). Using antibodies raised against this peptide the distribution of immunoreactive substance P was studied with the indirect immunofluorescence technique of Coons and collaborators Extensive neuronal systems containing substance P-like peptide were found within the brain spinal cord and periphery (see Hokfelt et al 1977) One such substance P system is represented by primary sensory neurons with cell bodies in spinal ganglia and central branches in the dorsal horn of the spinal cord and peripheral branches in many peripheral tissues including skin (Hokfelt et al 1977) A substance P like peptide has also been demonstrated by immunohistochemistry in some sensory neurons of the nodose ganglion of the vagus system (Lundberg et al 1978) In the present study further sensory system has been investigated gustatory nerves in the cat tongue Evidence presented that numerous substance P immunoreactive fibres are present in the taste buds

Four adult cats were perfused with ice cold 10% formalin for 30 min The tongue was dissected out and immersed in the same fixative for 90 min. After rinsing in 0 1 M phosphate buffer with 5% sucrose added for at least 4 h pieces of the tongue including the papillae vallatae were cut on a cryostat and the sections were incubated with substance P antiserum in a humid atmosphere at 4°C for 18–24 h. After rinsing the sections were incubated with fluoresceinisothiocyanate (FITC) conjugated sheep antirabbit antibodies for 30 min at 37°C rinsed mounted and examined in a Zeiss fluorescence microscope Substance P antiserum pretreated with an excess of substance P served as control serum.

In frontal sections of the papillae vallatae numerous substance P immunoreactive fibres could be seen within the connective tissue of the papillae (Fig. 1 A, B). The highest concentrations of fluorescent fibres were seen in the peripheral parts of the papillae close to the epithelium The fibres formed small bundles and often penetrated into the epithelium (Fig. 1 B). Particularly in the basal part of the papillae numerous taste buds could be observed. In all of these buds substance P immunoreactive fibers could be seen (Fig. 1 A, B) Their number as seen in a single section varied and could range from few fibers up to a dozen ones. They were slightly varicose and were mostly running towards the pore of the taste bud. Only few substance P immunoreactive fibres were seen in regions of the tongue outside the vallate papillae No such fibres could be seen after incubation with control serum.

The present findings indicate that a substance P-like peptide is present in nerve terminals within the taste buds in the papillae vallatae of cat tongue The fibres are mostly numerous and seem to be present in all taste buds. They therefore presumably represent the peripheral branches of gustatory nerves. Since the circumvallate papillae mostly are innervated by the glossopharyngeal nerve, it is interesting to note that in the rat and guinea-pig substance P-like immunoreactivity is present in gan-

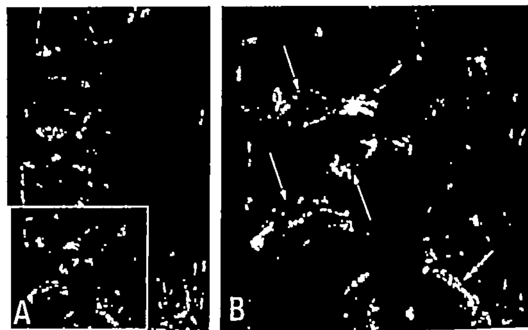


Fig 1 A B Immunofluorescence micrographs of the basal part of a cat vallate papillae after incubation with substance P antiserum B represents a higher magnification of part of A as indicated by rectangle. Numerous taste buds are seen, all of which contain substance P immunoreactive fibres (arrows) Note bundles of fluorescent fibres (double arrow) also in the connective tissue under the epithelium Magnifications ×130 (A) and ×350 (B).

glion cells of the petrosal ganglion (Lundberg un published data) as well as in the entire nucleus tractus solitarii (Ljungdahl et al 1978) The latter brain area and the petrosal ganglion are now studied in the cat with regard to possible occurrence of substance P-like immunoreactivity

The possible functional role of substance P in presumable gustatory neurons is not known It has been suggested that substance P may act as a neurotransmitter released in the spinal cord from the *central* branches of primary sensory neurons (Lembeck 1953 Otsuka & Takahashi 1977) and it may have a similar function in the gustatory neurons Of special interest is however the occurrence of the peptide in the *peripheral* branches within the taste buds since evidence is now accumulating that substance P may exert an action also peripherally Thus substance P may be the agent released to cause the vasodilation induced by antidromic stimulation (Hinsey & Gasser 1931) In fact Olgarth et al (1977) have shown that substance P can be released by nervous stimulation from presumable peripheral sensory branches in the tooth pulp Furthermore it has recently been demonstrated that more than 90% of the substance P immunoreactive material produced in sensory ganglia (nodose ganglion) is transported into the peripheral branches (Brimijoin et al 1980) A possible function of substance P in the periphery should therefore be seriously considered

It is well known that structural integrity of the taste buds is dependent on their sensory innervation (Olmsted 1970) The taste buds disappear after transection of the gustatory nerves but reappear when reinnervated by gustatory nerve fibres (Guth 1958) The differentiation of the taste buds is under the influence of a neurotrophic substance Torrey 1934) Also non-gustatory nerves can cause reappearance of taste buds (Poritsky & Singer 1963) Transplantation experiments to the anterior eye chamber have shown that different types of sensory ganglia can induce taste bud formation (Zalewski 1974) and that even in vallate papillae grafted alone to the anterior eye chamber reformation of taste buds can occur (Gómez Ramo et al 1979) It is interesting to note that sensory branches of substance P neurons are available in all models discussed above in pinal sensory ganglia (Hokfelt et al 1977) in the nodose ganglion (Lundberg et al 1978) and as free nerve endings in the iris (Hokfelt et al 1977) Could it be that substance P or a factor present in the same neuron represent the neurotrophic factor responsible for the structural integrity of taste bud Further functions should however

be considered Thus, substance P could e g. modulate the threshold of excitability of "its own" nerve ending or of the taste cells.

In conclusion, the present and earlier findings suggest that a substance P-like peptide is present in several types of sensory systems and may be involved in different sensory modalities such as pain visceral sensation and taste.

The present study was supported by grants from the Swedish Medical Research Council (04X 2887 04X 4495), Magnus Bergvalls Stiftelse and Knut och Alice Wallenbergs Stiftelse.

We thank Professor Yngve Zotterman for many valuable suggestions and discussions on topics related to taste and sensory mechanisms.

We are grateful to Mrs W Hiort and Miss U Lindefeldt for skilful technical assistance and Mrs Birgit Fridéen for excellent secretarial help

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Acta Physiol Scand 1979 107 393–395

Evidence for a dopaminergic pathway in the rat descending from the A11 cell group to the spinal cord

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It was suggested several years ago by Magnusson (1973) that part of the dopamine in the spinal cord serves an independent, non-precursor role. The existence of dopamine nerve fibers within the spinal cord has further been corroborated by the studies of Commissiong et al (1978, 1979) on the basis of biochemical experiments as well as of lesion studies. Very recently Blessing & Chalmers (1979) have demonstrated that in the rabbit hypothalamic catecholamine neurons of the A13 cell group of Fuxe (1965) project to the thoracic part of the spinal cord. Their evidence rests on a technique combining retrograde peroxidase tracing with catecholamine histochemistry In the present preliminary paper we report with a similar combination of retrograde tracing and transmitter immunohistochemistry the projection of dopaminergic A11 cells to the spinal cord of the rat.

Male specific pathogen-free Sprague-Dawley rats were used (b w 150 g) The fluorescent dye primuline (Kuypers et al. 1977) dissolved in distilled water (10% w/) was injected into the spinal cord at the lower thoracic and upper lumbar level (two bilateral injections of each 1 μl were made) After 4–10 days the rats were perfused with ice-cold formalin, and the brain and pinal cord were dissected out and immersed in the same fixative for 90 min After rinsing in 0 1 M phosphate buffer containing 5% sucrose for at least 4 h frontal sections of the hypothalamus and the rostral mesencephalon were cut on cryostat (section thickness 15 μm) The sections were mounted in a mixture of glycerin and phosphate buffer (3 1) and examined in a fluorescence microscope Cells containing clear yellow fluorescent granules wer photographed The cover slip was then removed and the sections were processed for the indirect immunofluorescence technique of Coons and collaborators (see Coons 1958). Briefly the sections were incubated with antiserum to tyrosine hydroxylase diluted 1 100 rinsed incubated with fluoresceinisothiocyanate (FITC) (SBL, Stockholm, Sweden) conjugated sheep antirabbit antibodies, rinsed mounted in a mixture of glycerin and phosphate buffer (3 1) and examined again in the fluorescence microscope Cells containing both the yellow fluorescent granules and tyrosine hydroxylase-like immunoreactivity were photographed again The technique described above is based on studies on retrogradely transported fluorescent dyes carried out by Kuypers and collaborators (Kuypers et al. 1977) and has been described in detail previously (Hökfelt et al 1979*b* *c*) The enzyme tyrosine hydroxylase is a marker for catecholamine neurons in general To differentiate between dopamine and noradrenaline neurons, adjacent sections were processed with antiserum against dopamine-β-hydroxylase a marker for noradrenaline (and adrenaline) neurons. Normal rabbit serum was used as control serum.

After injection of primuline into the spinal cord numerous intensely yellow fluorescent granules were found in cells in many areas of the hypothalamus (e.g. the paraventricular nucleus) and the mesencephalon (e g. the nucleus ruber) Of particular interest was a group of cells in the periventricular area at the border between the mesencephalon and the hypothalamus These cells were located in the grey matter around the third ventricle medial to the fasciclus retroflexus (Fig. 1 A) and their fluorescent granules were present in the perinuclear cytoplasm but extended often also into the cell processes (Fig. 1 A) After incubation with tyrosine hydroxylase antiserum, but not after incubation with dopamine β-hydroxylase antiserum, numerous cells in the same area exhibited the characteristic green fluorescence induced by FITC (Fig. 1B) The cells were often multipolar

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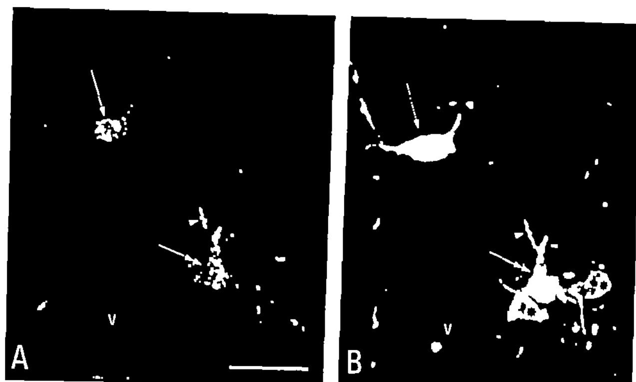


Fig 1A B Fluorescence micrographs of the periventricular grey at the border between mesencephalon and hypothalamus after injection of primuline into the spinal cord (A) and after incubation of the same section with tyrosine hydroxylase antiserum. Two cell bodies (arrows) contain both primuline (fluorescent granules in A) and tyrosine hydroxylase (B) The granules are mostly obscured by the immunofluorescence in B but they can be seen in the process indicated by arrow heads Two cells (asterisk) are only tyrosine hydroxylase immunoreactive *V* indicates a blood vessel Bar indicates 50 μm

with a size of about 30–40 μm and their localization corresponded well to that of the so-called A11 catecholamine group described by Dahlstrom & Fuxe (1964) Several of these cells also contained the yellow fluorescent granules which easily could be distinguished from the clear green FITC induced immunofluorescence particularly after prolonged exposure to UV light which induced a fading of the immunofluorescence No yellow fluorescent granules could be observed in tyrosine hydroxylase immunoreactive cells located more rostrally in the zona incerta belonging to the A13 cell group (Fuxe 1965) No dopamine β-hydroxylase immunoreactive cell bodies were observed in the adjacent sections and no fluorescent cells were seen after incubation with control serum

The present findings give evidence for a direct descending projection to the spinal cord from periventricular cells localized at the level of the posterior hypothalamus and the rostral mesencephalon and thus confirms the existence of a hypothalamo-spinal catecholamine system first described by Blessing & Chalmers (1979) The dopaminergic nature of these cells is indicated by their content of the enzyme tyrosine hydroxylase but the apparent lack of the noradrenaline synthesizing enzyme dopamine β-hydroxylase in agreement with previous immunohistochemical studies (Swanson & Hartman 1975 Hökfelt et al 1979*a*)

The pathway described by Blessing & Chalmers (1979) originate in the A13 cell group i e in the zona incerta rostral to the A11 group This discrepancy may be due to the fact that different species rat and rabbit have been studied Furthermore more extensive studies in our laboratory may be necessary to establish an additional projection also from the A13 group Commissiong et al (1979) have shown a decrease of dopamine in the spinal cord after lesion of substantia nigra. Thus there may exist several descending dopaminergic projections to the spinal cord On the other hand it must be clarified whether or not the lesions made by Commissiong et al (1979) possibly could interfere with the descending projection from the more rostrally located A11 dopamine cells

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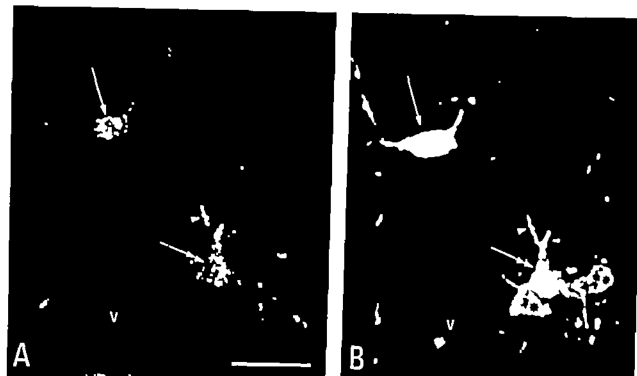


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Acta Physiol Scand 1979 107 397 399

Evidence for a selective reduction of adrenaline turnover in the dorsal midline area of the caudal medulla oblongata of young spontaneous hypertensive rats

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The adrenaline pathways innervate the cardiovascular areas of the brain (Hokfelt et al. 1973) and it has been proposed that the adrenaline neurons represent important central vasodepressor systems (Fuxe et al 1975) Increases in PNMT activity and changes in adrenaline levels have been observed in young and adult spontaneous hypertensive animals (Saavedra et al 1976, Sauter et al 1977 Wijnen et al 1978 Saavedra 1979) Recently it has been found that adrenaline turnover is reduced in the cardiovascular areas of the medulla oblongata in adult spontaneous hypertensive animals (Fuxe et al. 1979). Therefore in the present study adrenaline turnover has also been analyzed i young spontaneous hypertensive animals to evaluate if the reduction of adrenaline turnover is primary or secondary to the hypertensi e process.

Female, 4-week old SH rats of the stroke prone strain (sp-SH) ere compared with normotensive Wistar Kyoto (WKy) control rats (Okamoto et al 1974), which were originally derived from the Wistar Kyoto strain (Okamoto 1972) and bred for 4 years at the department of Pharmacology University of Heidelberg Tail cuff plethysmography was used to measure systolic arterial blood pressure The rats were given food pellets and water ad libitum and re kept on regular day and night schedule (lights on at 6 m and off at 8 p m) They were killed by decapitation between 9 and 12 m. Adrenaline turnover in the hypothalamus and in the dorsal midline area of the caudal medulla oblongata (DCMO) were measured by studying the disappearance of the adrenaline stores using inhibitors of dopamine-β-hydroxylase (FLA 63 (bis-(4-methyl-1-homopiperazinyl-thiocarbonyl)-disulphide, 10 mg/kg, p 2 h before killing) and of phenylethanolamine-N-methyltransferase, SK&F 64139 (7,8-dichloro-1,2,4-tetrahydroisoquinoline hydrochloride 40 mg/kg, p 2 h before killing) (Corrodi et al. 1970, Pendleton et al. 1976). The hypothalamus and the DCMO were punched out from frozen coronal sections of the diencephalon and the medulla oblongata respectively DCMO contains inter alia the nuc. tractus solitarius, nuc. dorsalis motorius nervi vagi and parts of the nuc. commissuralis (see Fuxe et al. 1979). The catecholamine levels were determined by means of high pressure liquid chromatography in combination with electro-chemical detection, using α-methyl dopamine as an internal standard (Keller et al 1976).

The arterial blood pressure in the young sp-SH rats was 117±3.2 ($n=13$) mmHg and in the normotensive WKy rats 96.5± 1 ($n=13$) mmHg. This difference was significant ($P<0.01$ Student's t-test for unpaired data).

As seen in Table 1 the hypothalamic dopamine and noradrenaline levels were significantly reduced and increased respectively in the 4-week old spontaneous hypertensive rats, and there was also a trend for an increase of the adrenaline stores in the hypothalamus. The dopamine noradrenaline and adrenaline levels in the DCMO were however not changed in the sp-SH rats compared with normotensive WKy rats. In Table it is demonstrated that a marked reduction of the FLA 63 and SK&F 64139 induced adrenaline disappearance occurs in the DCMO of 4 week old sp-SH rats compared with normotensive WKy controls. The disappearance of adrenaline stores in the hypothalamus following amine synthesis inhibition is not changed in sp-SH rats versus WKy rats Furthermore the FLA 63 induced noradrenaline disappearance in the hypothalamus and the DCMO is not significantly different in the sp-SH rats versus the WKy controls.

taneous hypertension but appear to increase their contents of adrenaline in agreement with previous work (Gianutsos & Moore 1978, Wijnen et al. 1978). It is likely that the differential regulation of medulla oblongata and hypothalamic adrenaline terminals is due to the fact that adrenaline terminals in the hypothalamus not only subserve cardiovascular functions but also neuroendocrine and behavioural functions (Hökfelt et al. 1973, Wijnen et al. 1978). Furthermore, the present findings do not support the view that the noradrenaline systems within the hypothalamus and the DCMO play a major role in the development of spontaneous hypertension. It seems possible that the increase of hypothalamic noradrenaline levels found in the sp-SH rats could be secondary to the marked reduction of adrenaline activity in the brain stem, especially since treatment with SK&F 64139 increased the noradrenaline levels in the normotensive WKy animals alone. The mechanism for the reduction in hypothalamic dopamine levels in young sp-SH rats remains to be elucidated. In conclusion the present results indicate that adrenaline turnover is markedly reduced in the DCMO of young female sp-SH rats versus WKy control rats, which may lead to reduced activity in the central baroreceptor reflex pathway and thus contribute to the development of the hypertension.

This work was supported by a grant (04X-4626) from the Swedish Medical Research Council and by a grant from Magnus Bergvall's Stiftelse.

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Table 1 *Catecholamine levels in the hypothalamus and the dorsal part of the caudal medulla oblongata (DCMO) of young sp-SH and WKy rats*

Means±S.E. are shown. *n*=number of animals. The values are given as ng/g wet weight. The statistical comparisons have been made between the corresponding values in the WKy and sp-SH rats using Student's *t*-test for unpaired data. NS=not significant

	Hypothalamus				DCMO			
	WKy		sp-SH	*n*	WKy		sp-SH	*n*
DA	573±34	$P<0.05$	464±[illegible]0	7–9	[illegible]07±[illegible]8	NS	191±[illegible]1	5–7
NA	1 858±91	$P<0.025$	2 146±60	7–9	2 107±[illegible]63	NS	1 628±260	5–7
A	58± 5	$P=0.1$	75± 8	7–8	48± 9	NS	50± 10	5–7

Table 2 *Per cent depletion of NA and A stores in hypothalamus and DCMO of young sp-SH and WKy rats following treatment with FLA 63 and SK&F 64139*

The CA levels are given in per cent of the CA levels of untreated WKy and sp-SH rats respectively (for absolute levels, see Table 1). Means ± S.E. *n*=number of rats. Student's *t* test. For further details, see Table 1 and text

Treatment	CA	CA levels in % WKy	Statistical significance WKy vs. sp-SH	CA levels in % sp.SH	*n*
Hypothalamus					
No drug treatment	DA	100± 6		100± 4	7–9
FLA 63	DA	134± 7	$P<0.05$	178±13	8
SK&F 64139	DA	119± 5	NS	113± 7	8
No drug treatment	NA	100± 5		100± 5	7 9
FLA 63	NA	69± 6	NS	57± 3	8
SK&F 64139	NA	116±6	$P<0.05$	100± [illegible]	8
No drug treatment	A	100± 9		100± 8	7–8
FLA 63	A	75±1	NS	56± 4	8
SK&F 64139	A	66± 3	NS	61±11	7
DCMO					
No drug treatment	DA	100±14		100±11	5–7
FLA 63	DA	[illegible]04±27	NS	164±[illegible]0	5–7
SK&F 64139	DA	1[illegible]1±[illegible]2	NS	1[illegible]0±10	6–8
No drug treatment	NA	100±13		100±16	5–7
FLA 63	NA	50± 4	NS	7[illegible]± 9	5–7
SK&F 64139	NA	100± 5	NS	108± 9	6
No drug treatment	A	100±19		100± 0	5–7
FLA 63	A	31± 4	$P<0.001$	86± 8	5–7
SK&F 64139	A	39± 6	$P<0.001$	100± 8	5–8

However, treatment with SK&F 64139 results in an increase of the hypothalamic noradrenaline stores of the WKy control rats, an effect which is not observed in the sp-SH rats.

The present paper gives evidence for a marked reduction of adrenaline turnover in the DCMO of young female spontaneous hypertensive rats compared with appropriate normotensive WKy controls at a time when the increase in the arterial blood pressure begins to develop in the sp-SH rats. These results support the hypothesis that *adrenaline nerve* terminals in the cardiovascular areas of the medulla oblongata represent an important vasodepressor mechanism (Fuxe et al. 1975) which can be pathologically disturbed in animals which will develop spontaneous hypertension. It seems possible that such a disturbance can be one of the factors producing spontaneous hypertension. The hypothalamic adrenaline nerve terminals seem to be independently controlled from the DCMO adrenaline terminals. Thus they do not change their turnover in relation to the development of spon-

neous hypertension but appear to increase their contents of adrenaline in agreement with previous work (Gianutsos & Moore 1978, Wijnen et al. 1978). It is likely that the differential regulation of medulla oblongata and hypothalamic adrenaline terminals is due to the fact that adrenaline terminals in the hypothalamus not only subserve cardiovascular functions but also neuroendocrine and behavioural functions (Hokfelt et al. 1973, Wijnen et al. 1978). Furthermore, the present findings do not support the view that the noradrenaline systems within the hypothalamus and the DCMO play a major role in the development of spontaneous hypertension. It seems possible that the increase of hypothalamic noradrenalin levels found in the sp-SH rats could be secondary to the marked reduction of adrenaline activity in the brain stem, especially since treatment with SK&F 64139 increased the noradrenaline levels in the normotensive WKy animals alone. The mechanism for the reduction in hypothalamic dopamine levels in young sp-SH rats remains to be elucidated. In conclusion, the present results indicate that adrenaline turnover is markedly reduced in the DCMO of young female sp-SH rats versus WKy control rats, which may lead to reduced activity in the central baroreceptor reflex pathway and thus contribute to the development of the hypertension.

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